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14. ABSTRACT

Scope: Prostate cancer (CaP) is characterized by unique prostate-associated antigens; hence, it has been considered a prime candidate for immunotherapy. Despite numerous laboratory advances, clinical outcomes have been partial and transient.

Purpose: The overall goal of the proposed studies is to optimize the effectiveness of therapeutic whole-cell CaP vaccines by taking into consideration tumor-associated hypoxia as a relevant determinant of tumor antigenicity.

Major findings: Transcriptome studies revealed that gene expression in hypoxically cultured cells is more akin to that in tumor cells *in situ* than are cells grown normoxically. Transcripts of hypoxia-associated genes DLG7, CCNB1 and HMMR were associated with Gleason score and with disease prognosis suggesting their potential as CaP biomarkers with prognostic value. By 2D-gel electrophoresis, we screened patient sera and detected novel hypoxic-cell reactive autoantibodies (under validation).

Significance: Our data suggest that hypoxically cultured CaP cells are more akin to tumor cells *in situ* than are cells grown normoxically. We have identified hypoxia-reactive proteins, pathways and autoantigens with potential value as biomarkers or therapeutic targets. Introduction of pO_2 as a variable can constitute a tool for the development of more effective immunotherapy for CaP.

15. SUBJECT TERMS

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Introduction

Prostate cancer (CaP) remains among the most common causes of cancer-related deaths in men. Because CaP is characterized by unique prostate-associated antigens, it has been considered among prime candidates for immunotherapy. Despite numerous laboratory advances, clinical outcomes have been partial and transient. One plausible reason for the incomplete response is that vaccine cells, prepared under standard tissue culture conditions, can drastically differ in expression of macromolecules *in situ*, and thus may immunize against a less complete antigen spectrum. The purpose of the proposed studies is to optimize the effectiveness of therapeutic whole-cell CaP vaccines by taking into consideration tumor-associated hypoxia as a relevant determinant factor of tumor antigenicity. We hypothesize that hypoxically cultured CaP cells are more similar in their antigen landscape to CaP cells *in situ* than are normoxically cultured CaP cells. The following Tasks were defined in the approved statement of work; Task 1. Identify oxygen-tension responsive genes and proteins in the cells comprising a clinical-grade CaP cellular vaccine. This first task was accomplished at the end of the first period. Task 2. Validate differentially expressed molecules in CaP that are associated with tissue hypoxia. This last task has been partially executed and is reported in the present progress report. If the proposed studies demonstrate that CaP cells grown under low oxygen tension (pO_2) are more antigenically similar to cells *in situ*, this will justify the evaluation of their therapeutic value in a preclinical model.

Body

Task 1. Identify oxygen-tension responsive genes and proteins in the cells comprising a clinical-grade CaP cellular vaccine (Completed)

Approach: Identification of specific candidate genes with pO_2 -dependent expression in CaP cells has not been established yet in the context of their antigenic relevance. In Task 1, CaP cells grown at different pO_2 were tested by state-of-the-art high throughput genomics and proteomics techniques. This approach was designed to identify pO_2 -regulated tumor-associated pathways and macromolecules. To determine the antigenic potential of pO_2 -regulated tumor-associated macromolecules, we tested their reactivity with the spontaneous antibodies from CaP patients and the sera of age-matched non-cancerous controls, other cancers and an autoimmune disease.

Summary of key research accomplishments related to Task 1 (as they relate to the proposed sub Tasks):

Task 1a. To propagate LnCaP and VCaP cells under pO_2 -controlled conditions:

- Hypoxic LnCaP and VCaP cells proliferate more effectively than at standard cell culture conditions.
- Hypoxic cells secrete more VEGF.

Task 1b. cDNA gene microarrays and data analysis:

- Hypoxia induced overexpression of molecules involved in intracellular signaling networks in cancer and in urologic diseases in comparison to normoxic cells.
- Hypoxia increased transcript levels for some genes in cell lines to levels comparable to those in CaP tissue.
- Hypoxia-associated the disc large (Drosophila) homolog-associated protein 5 [DLG7], cyclin B1 [CCNB1], and hyaluronan-mediated motility receptor [HMMR] genes were

significantly overexpressed in CaP and were associated with Gleason score and disease prognosis.

- Hypoxic cells expressed 30 to 60 percent more CCNB1 and DLG7 transcripts.

Task 1c. 2-D gel analysis, in gel enzyme digestion and mass spectrometry:

- The change in pO_2 , affected the proteome mostly quantitatively (*i.e.*, by change in spot intensity).

Task 1d. Association between gene-specific changes in mRNA and hypoxic proteome:

- There was no correlation between changes in protein levels and mRNA induction among a group of select genes tested.

Task 1e. Identification of CaP antigens by 2D–Western blots

- Protein lysates from cells exposed to hypoxia revealed novel potential tumor associated antigens (TAAs) (currently under validation) in sera from CaP patients (heat shock 70 kDa protein 4; protein disulfide isomerase A3; heterogeneous nuclear ribonucleoprotein L; U1 small nuclear ribonucleoprotein 70kDa and leucine-rich repeat-containing protein 47).

Task 2. Validate differentially expressed molecules in CaP in association with tissue hypoxia

Approach: The presence of a hypoxic cancer microenvironment correlates with increased tumor invasiveness, metastases, resistance to radio- and chemotherapy, and poor clinical outcome (Vaupel, Kelleher et al. 2001; Overgaard 2007). It is well established that CaP cells are found under hypoxic conditions *in vivo* (Movsas, Chapman et al. 2001) and that numerous proteins are modified in their expression by hypoxia (Koritzinsky, Seigneure et al. 2005). Although many endogenous markers have been associated with the hypoxia response in cancer they are not all unregulated in primary CaP tissue (Stewart, Gray et al. 2008). This may be because the evaluation of potential markers has not been made taking into consideration the hypoxic environment in first place. Task 2 (planned for the second period of the award) is aimed at assessing the expression of select candidate genes identified in Task 1 in CaP tissue. Real time PCR and RNA *in situ* hybridization added to immunodetection will allow detection of specific candidate genes in CaP tissue.

The proposed sub Tasks are:

2a. RNA extraction and real time quantitative PCR in CaP tissue (months 13-16)

2b. mRNA *in situ* hybridization (months 16-22)

2c. Immunohistochemistry Staining in CaP tissue (months 16-24)

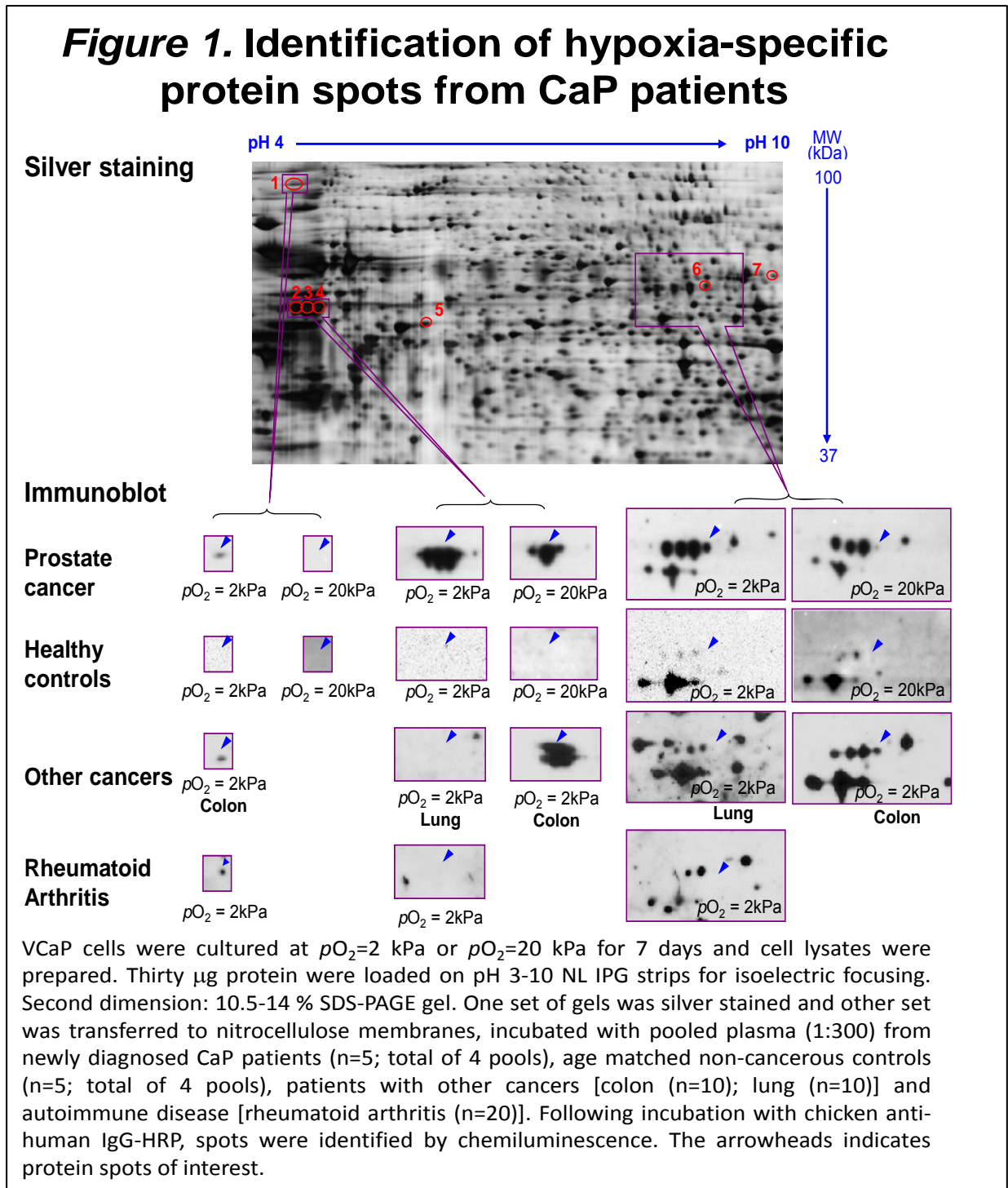
Note: Because of my recent relocation to the University of Mississippi Medical Center (UMMC) in September 2011, the timing for accomplishing Task2, originally proposed for year 2 (last period for this award) needed to be readjusted. For this purpose, I requested a no-cost extension to the PCRP-CDMRP. Approval was received on May 2012, extending the period of performance from: 15 May 2010 - 14 June 2012 (research ends 14 May 2012) to 15 May 2010 - 14 June 2013 (research ends 14 May 2013). Appended is the fully executed modification of the basic award. With this modification it will be possible to complete the remaining sub Tasks.

In Task 1e we studied the reactivity of autoantibodies in CaP patient plasma. Following published protocols (Desmetz, Bibeau et al. 2008), we prepared total cell lysates of CaP cells (VCaP and LnCaP) cultured at $pO_2=2$ kPa or 20 kPa, resolved them by 2D electrophoresis, transferred onto nitrocellulose membranes. Total cell lysates were incubated with pooled plasma (1:300) from newly diagnosed Gleason 6 CaP patients (n=5; total of 4 pools), age matched non-cancerous controls (n=5; total of 4 pools), patients with other cancers [colon (n=10); lung (n=10)] and autoimmune disease [rheumatoid arthritis (n=20)]. Autoantibodies from CaP patient plasma specifically bound to seven spots; four were hypoxia-specific. All selected spots were excised from the gel, trypsin-digested, and analyzed by MALDI-TOF mass spectrometry (**Table 1**). We identified them as heat shock 70 kDa protein 4; 60 kDa heat shock protein; protein disulfide isomerase A3; heterogeneous nuclear ribonucleoprotein L; U1 small nuclear ribonucleoprotein 70kDa and leucine-rich repeat-containing protein 47. With the exception of the latter molecule, identified proteins have been identified or validated as TAAs before [see references in **Table 1**]. However, to the best of our knowledge none of the proteins has been validated as a TAA in CaP.

Table 1. Potential TAAs identified in VCaP cells						
Spot number	Protein name	Hypoxia specific	Accession number	MW (kDa)	Peptides matched	TAA [Refs]
1	Heat shock 70 kDa protein 4	yes	P34932	94.3	28-39	Esophageal (Zhang, Wang et al. 2011), hepatocellular carcinoma (Takashima, Kuramitsu et al. 2006; Looi, Nakayasu et al. 2008)
2	60 kDa heat shock protein	no	P10809	61.3	43-63	Breast (Desmetz, Bibeau et al. 2008), hepatocellular carcinoma (Looi, Nakayasu et al. 2008), colorectal (He, Wu et al. 2007), oral (Castelli, Cianfriglia et al. 2001), gastric lymphoma (Takenaka, Yokota et al. 2004)
3	60 kDa heat shock protein	no	P10809	61.3	59-80	
4	60 kDa heat shock protein	no	P10809	61.3	50-105	
5	Protein disulfide isomerase A3	yes	P30101	56.8	16-26	Breast (Desmetz, Bibeau et al. 2008; Hamrita, Chahed et al. 2008), hepatocellular carcinoma (Looi, Nakayasu et al. 2008)
6	Heterogeneous nuclear ribonucleoprotein L	yes	P14866	64.1	18-52	Acute leukemia (Cui, Li et al. 2005), healthy (Li, Zhao et al. 2006)
6	U1 small nuclear ribonucleoprotein 70kDa	yes	P08621	51.4	11-52	Lymphoma (Cha, Kwak et al. 2006)
7	Leucine-rich repeat-containing protein 47	yes	Q8N1G4	63.5	16-16	

Identification of hypoxia-sensitive TAAs from CaP patients: Among the most conspicuous potential TAAs recognized by plasma from CaP patients in VCaP lysates by 2D–Western blots were spots 1; 2-4; and 6 (**Table 1**). Spot 1, a hypoxia-sensitive spot, contains the Heat shock 70 kDa protein 4 (HSP70). This protein has been identified as TAA in esophageal (Zhang, Wang et al. 2011), and hepatocellular carcinoma (Takashima, Kuramitsu et al. 2006; Looi, Nakayasu et

al. 2008). Protein spot 1 exhibited no reactivity with sera of healthy controls (**Figure 1**), but was reactive to colon cancer and rheumatoid arthritis, thus anticipating lack of tumor specificity. Interestingly, the sequence of spots 2, 3 and 4 corresponded to the 60 kDa heat shock protein, identified by us as a hypoxia-insensitive TAA. As there are three Hsp60 isoforms (Raulston, Paul et al. 1998), it is likely that we identified the three isoforms as potential TAAs. Protein spots 2, 3 and 4 exhibited no reactivity with sera of healthy controls, lung cancer and rheumatoid arthritis (**Figure 1**). We detected, in agreement with published data (He, Wu et al. 2007), some



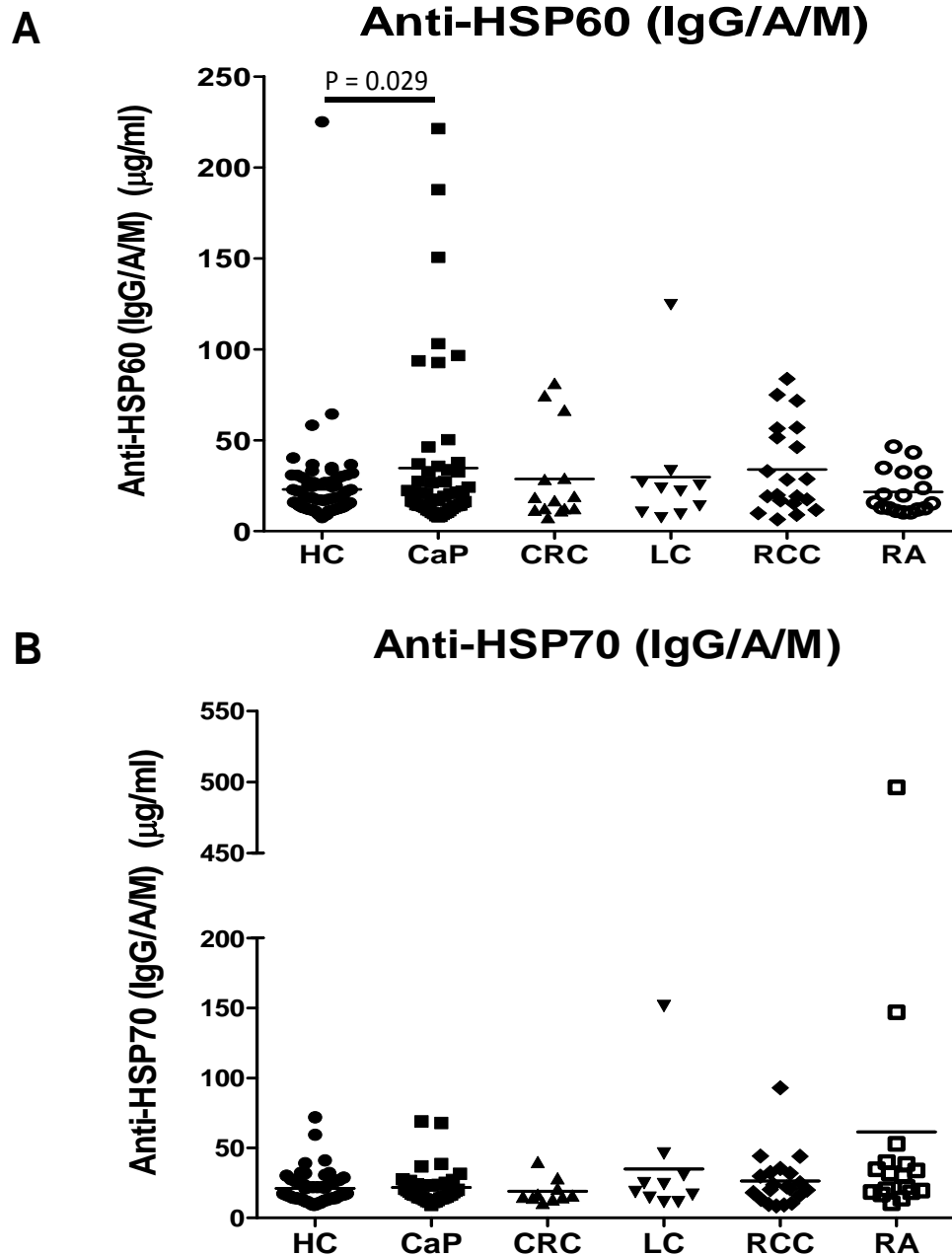
reactivity (in a hypoxia-dependent manner) with plasma from colorectal cancer (CRC) patients. The relevance of hypoxia has been recognized in CRC (Waldner and Neurath 2010) and numerous CRC-associated-TAAs have been identified (Reuschenbach, von Knebel Doeberitz et al. 2009); however, establishing the relevance of hypoxia-sensitive HSP60 is a new and interesting aspect in CRC; as it could expand the use of hypoxia to the identification of TAAs in other tumors.

Spot 6; contains the U1 small nuclear ribonucleoprotein 70kDa and the heterogeneous nuclear ribonucleoprotein L (hnRNP L). Both of these proteins have been identified as TAAs (Cui, Li et al. 2005; Cha, Kwak et al. 2006; Li, Zhao et al. 2006). Additional sequencing in LnCaP cells (data not shown) suggested hnRNP L as the candidate of interests for further validation. Protein spot 6 exhibited little or no reactivity with sera of healthy controls, lung cancer or rheumatoid arthritis (**Figure 1**), but we detected some reactivity (in a hypoxia-dependent manner) with plasma from CRC patients. By similar reasons to those discussed for HSP60, establishing the relevance of hypoxia-sensitive hnRNP L could be rewarding, especially in regards to its identification as a potential TAA.

Frequency of Autoantibodies in the sera of CaP patients: We performed ELISA tests to determine the frequency of autoantibodies for two of the three selected potential TAAs identified by 2D–Western blots: HSP60 and HSP70. The sera of CaP patients (n=54), healthy controls [males (n=76); females (n=6)], colorectal cancer (n=14), renal cell carcinoma (n=20), lung cancer (n=10), and rheumatoid arthritis (n=17). The frequencies of IgG/A/M for HSP60 were quantified using commercial kits [Anti-HSP60 IgG/A/M (cat# ADI-EKS-650) and Anti-HSP70 IgG/A/M cat# ADI-EKS-750] from Enzo Life Sciences (Plymouth Meeting, PA). This assay allows for reproducible, accurate, and precise determination of IgG, IgA and IgM antibodies (total) to the analyte of interest in plasma. Recombinant human protein (e.g. HSP60 or HSP70) is bound to the wells of the plate to bind anti-human antibodies for the analyte of interest present in plasma. The captured anti-human antibodies are detected with a HRP conjugated goat polyclonal antibody specific for human IgG, IgA, and IgM molecules. The results are expressed in proportion to the amount of captured anti-human antibodies. The frequency of HSP60 autoantibodies (**Figure 2A**) were 13.0% (7/54) in CaP (average \pm SD = 34.67 ± 43.94 μ g/ml), compared to 1.3% (1/76) in healthy controls (average \pm SD = 22.96 ± 25.77 μ g/ml); 7.14% (1/14) in colorectal cancer (average \pm SD = 28.66 ± 25.52 μ g/ml); 7.14% (1/10) in lung cancer (average \pm SD = 29.72 ± 34.42 μ g/ml); 7.14% (1/20) in renal cell carcinoma (average \pm SD = 33.82 ± 24.20 μ g/ml); and 0% (0/17) in rheumatoid arthritis (average \pm SD = 21.52 ± 11.93 μ g/ml). These data suggest that HSP60 autoantibodies may be elevated in the CaP patient plasma. Next, the frequency of HSP70 autoantibodies (identified as a hypoxia-specific TAA) was analyzed. TAAs frequency for HSP70 (**Figure 2B**) were 5.3% (2/38) in CaP (average \pm SD = 21.81 ± 13.06 μ g/ml), compared to 3.7% (2/54) in healthy controls (average \pm SD = 21.10 ± 11.80 μ g/ml); 7.14% (0/10) in colorectal cancer (average \pm SD = 19.01 ± 8.81 μ g/ml); 20.0% (2/10) in lung cancer (average \pm SD = 34.93 ± 42.21 μ g/ml); 5.0% (1/20) in renal cell carcinoma (average \pm SD = 26.40 ± 18.97 μ g/ml); and 17.65% (3/17) in rheumatoid arthritis (average \pm SD = 61.49 ± 116.3 μ g/ml). TAAs for HSP70 were not elevated in CaP patients relative to healthy controls; and even more, higher autoantibodies frequencies were found in other tumors and in rheumatoid arthritis (in agreement with data from 2D–Western blot), a non-cancer-related inflammatory disease. This result contrasts with previous findings suggesting HSP70 as a potential TAA in esophageal (Zhang, Wang et al. 2011), and hepatocellular carcinoma (Takashima, Kuramitsu et al. 2006; Looi, Nakayasu et al. 2008)

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Figure 2. Autoantibodies in the Plasma of CaP Patients



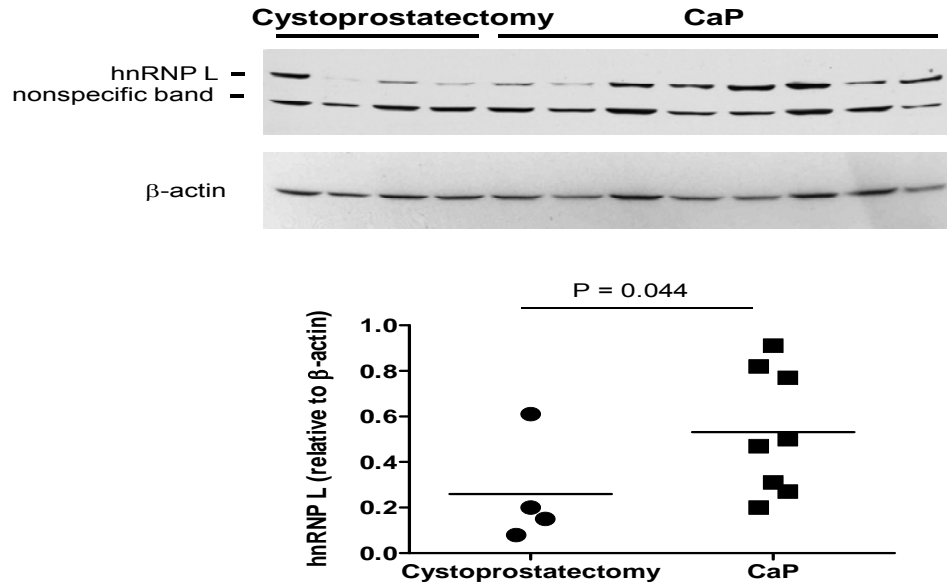
Autoantibodies to HSP60 (**Figure 2A**) and HSP70 (**Figure 2B**) were quantitated in the plasma of prostate cancer (CaP; $n = 54$); healthy controls (HC; $n = 76$); colorectal cancer (CRC; $n = 14$); lung cancer (LC; $n = 10$); renal cell carcinoma (RCC; $n = 20$); and rheumatoid arthritis (RA; $n = 17$). Plasma samples were diluted to 1/1000. The cutoff of reactivity was defined as the mean of sample plus 2 folds of standard deviation from normal plasma. Comparisons were done using t- test, one-tail. The level of significance was set at $P < 0.05$.

Overall, this data confirms HSP60 as a potential TAA in CaP. The results are in agreement with reported findings in other tumors (Castelli, Cianfriglia et al. 2001; Takenaka, Yokota et al. 2004; He, Wu et al. 2007; Desmetz, Bibeau et al. 2008; Looi, Nakayasu et al. 2008) demonstrating that HSP60 can elicit a humoral response. Our data represents the first one to suggest a specific humoral response against HSP60 in CaP. Further data evaluation in an independent validation group is needed to evaluate the performance and diagnostic value of HSP60 autoantibodies. For this purpose we are currently collaborating with Faculty at the Department of Urology and Pathology at UMMC. Blood and plasma samples are being collected from CaP patients recruited from Urology Clinics at the UMMC. An aliquot of plasma is secured for TAAs studies. Currently, we are in the process of acquiring the reagents for detecting antibodies to hnRNP L; its validation is critical in order to test the value of this, hypoxia-specific, as a potential TAA.

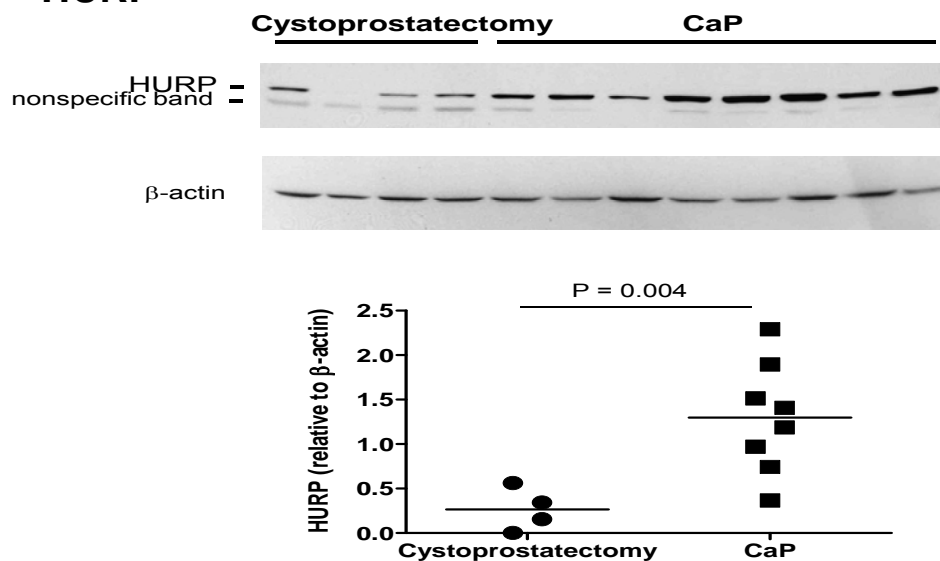
To examine the possibility that the reactivity of CaP sera is due to an elevated expression of selected TAAs, the mRNA expression of HSP60 and hnRNP L, if we validate it as a potential TAA candidate, will be analyzed. In addition, the expression and tissue localization for potential candidates will be evaluated in CaP patients. To progress with this Task, protein lysates were prepared from tumors obtained from eight CaP patients. Protein lysates from four control cystoprostatectomy patients were used as controls. Separated proteins were transferred onto a nitrocellulose membrane and blotted against an anti-human hnRNP L monoclonal antibody (Abcam, cat# ab6106). The results were normalized to beta (β)-actin (Novus, cat# NB600-501) and the ratio between hnRNP L and beta-actin was calculated to normalize the data. Normalized ratios were compared between controls and CaP tumors. The level of hnRNP L protein was significantly higher ($p < 0.05$); with a 2.04 fold increase compared to control prostate tissue (**Figure 3A**). This preliminary data suggests that protein expression for at least one of the selected potential TAAs under current validation may be elevated in CaP. Our next step will be to study gene expression in conjunction with a histopathology evaluation of CaP tissue. As part of the Prostate Cancer Foundation (PCF) awarded project “Hypoxia-regulated expression of DLG7 gene in prostate cancer prognosis and progression”, assessment the relationship of DLG7 expression and cancer-specific outcomes is a most relevant Task. We applied a similar strategy to explore the expression of the DLG7 gene product, hepatoma up-regulated protein (HURP) in CaP tissue with high RNA expression for DLG7, identified by cDNA microarrays. The level of HURP protein (detected by anti-HURP antibodies, cat# ab79870) was significantly higher ($p < 0.005$); with a 4.89 fold increase compared to control prostate tissue (**Figure 3B**); therefore, suggesting that gene and protein expression can be associated in our experimental groups. Analysis of the association of transcript and protein levels with clinical parameters will follow. In November 2011 the PI was offered a Research Collaborator position at the Department of Hematology, Mayo Clinic, Rochester, MN. The appointment allows ongoing funded research projects between his lab and Mayo Clinic researchers to continue, and to develop and maintain future investigative efforts. In relation to this project, validation of candidate TAAs in plasma and CaP tissue from patients belonging to the Mayo's prostate SPORC is in place. For this last purpose there is a currently active IRB protocol (Mayo IRB#10-000306). Approval for processing and transferring of frozen tissue for RNA analysis; and paraffin embedded tissue sections for immunostaining has been recently obtained (see attached minute excerpt from Mayo IRB). The analysis and interpretation of the generated data will be performed by Drs. George Vasmatazis, John Cheville, and Farhad Kosari at Mayo's Center for individualized Medicine. It is expected that the completion of Task 2 will occur as proposed.

Figure 3. Protein levels in CaP tumor lysates

A hnRNP L



B HURP



Protein lysates were prepared from prostate frozen tissue obtained from CaP patients (n=8) and cystoprostatectomy patients (n=4) used as controls. Thirty μ g protein were resolved in a 10.5-14 % SDS-PAGE gel; and transferred to a nitrocellulose membrane, blotted with anti hnRNP L (1/5000) (**Figure 3A**), HURP (1/100) (**Figure 3B**) and β actin (1/5000) antibodies. Following incubation with the respective IgG-HRP, bands were identified by chemiluminescence. The relative intensities of the bands were quantified using the Image J Software, and all the values were normalized to the intensities of the respective β -Actin signal. Results are expressed as the normalized optical density (OD) for individual patients. The mean OD for each experimental group is also shown. Comparisons were done the using the Mann–Whitney U test. The level of significance was set at $P < 0.05$.

Key research accomplishments

- Three potential TAAs; two hypoxia-specific (HSP70 and hnRNP L) and one hypoxia-nonspecific (HSP60) were selected for validation.
- The frequency of HSP60 autoantibodies was elevated in the CaP patient plasma.
- The frequency of HSP70 autoantibodies was not elevated in CaP patients relative to healthy controls; even more, higher autoantibodies frequencies were found in other tumors and in rheumatoid arthritis.
- Protein expression for hnRNP L (detected by Western Blot technique) was elevated in CaP tissue. Similarly, protein expression for HURP, another hypoxia-specific protein, was elevated in CaP tissue.

Reportable outcomes

Articles

1. **Gomez, C.R.**, Knutson, G., Bulur, P., Schreiber, C., Vuk-Pavlovic S. Age-dependent response of bone marrow cells to hyperbaric oxygen. BMC Medicine. DOI: 10.1007/s10522-012-9373-8
2. Nomellini, V., Mahbub, S., **Gomez, C.R.**, and Kovacs, E.J. Dysregulation of neutrophil CXCR2 and pulmonary endothelial icam-1 promotes age-related pulmonary inflammation. 2012. Aging Dis. 3:234-47

Abstracts

1. **Gomez, C.R.**, Kosari, F., Schreiber, C.A., Knutson G.J., Vasmatazsis, G., Vuk-Pavlović S. 2011. Hypoxic cell culture for more effective cancer vaccines. International Society for Applied Biological Sciences Conference on Forensic Genetics (ISABS). Bluesun hotel ELAPHUSA, Bol, Island Brač, Croatia
2. **Gomez, C.R.**, Munz, J.M., Kosari, F., Karnes R.J., Cheville, J., Ida, C.M., Sebo, T.J., Nair, A.A., Tang, H., Vasmatazsis, G., Vuk-Pavlović S. 2011. Prognostic value of hypoxia-controlled genes in high-risk prostate cancer. Eighteen Annual Prostate Cancer Foundation Scientific Retreat. Incline Village, Lake Tahoe, NV
3. **Gomez, C.R.**, Schreiber, C.A., Knutson G.J., Charlesworth, M.C., Vuk-Pavlović S. 2011. Targeting hypoxia for more effective immunotherapy of prostate cancer. Molecular Targets and Cancer Therapeutics AACR-NCI-EORTC International Conference. San Francisco, CA
4. **Gomez, C.R.**, Kosari, F., Munz, J.M., Schreiber, C., Knutson, G., Charlesworth, C., Karnes, R. J., Cheville, J., Vasmatazsis, G., Vuk-Pavlovic, S. 2012. Prognostic value of hypoxia-associated genes in prostate cancer. Advances in Prostate Cancer Research. Orlando, FL
5. **Gomez, C.R.**, Munz, J.M., Kosari, F., Karnes R.J., Cheville, J., Ida, C.M., Sebo, T.J., Vasmatazsis, G., Vuk-Pavlović S. 2012. Targeting hypoxia for the identification of novel biomarkers for prostate cancer. 103th AACR Annual Meeting. Chicago, IL

Funding Applied

1. Title: Mississippi Prostate Cancer HBCU Undergraduate Research Training Program. Program Coordinator and Mentor. Date: June 2012. Funding agency: Collaborative Undergraduate HBCU Student Summer Training Program Award from the Department of Defense; CDMRP, Prostate Cancer Research Program. Performance period: 04/01/13 – 03/30/15. Level of Funding: \$185,000. Status: Under review. Project goals: Aim 1: To

recruit 4 undergraduate trainees per year from Tougaloo College; Aim 2: To develop their skills through a comprehensive training curriculum in CaP research at UMMC-Cancer Institute and; Aim 3: To track and coach trainees on their progress towards becoming biomedical CaP researchers. Key personnel receiving salary support from this project: Srinivasan Vijayakumar, M.D. 0.12 Calendar months (Principal Investigator), Christian R. Gomez, Ph.D. 0.36 Calendar months (Program Coordinator and Mentor).

2. Title: Hypoxia enhances prostate cancer radioresistance by promoting cancer cell stemness. P.I. Date: March 2012. Funding agency: BD Biosciences Research Grant Program-Stem Cell Grant. Time commitment: 5%. Performance period: 07/01/12 – 07/01/14. Level of funding: \$10,000. Status: Not funded. Project goal: test the hypothesis that a low oxygen environment (where radiation is less effective due to the lack of the oxygen effect) increases the fraction of cancer stem cells in the population giving thus rise to a more resistant tumor. Key personnel receiving salary support from this project: 10% effort flow cytometry core personnel.
3. Title: A gene panel predictive of outcome in men at high-risk prostate cancer: Prognostic performance in African Americans. Funding agency: Bayer-Grants4Targets. Time commitment: 5%. Performance period: 06/01/12 – 06/01/14. Level of funding: \$150,000. Status: Not funded. Project goal: test the hypothesis that a panel of hypoxia-sensitive genes and TAAs identified in Caucasians has prognostic value in patients from other races with diagnosed CaP and in other cancers. Key personnel receiving salary support from this project: 100% effort postdoctoral fellow.

Research opportunities

In November 2011 the PI was offered a Research Collaborator position at the Department of Hematology, Mayo Clinic, Rochester, MN (see appended appointment notification letter). The appointment allows continuing ongoing funded research projects between his lab and Mayo Clinic researchers, and to develop and maintain future investigative efforts. One project [DOD PCRP W81XWH-10-1-0225, PI] needs validation of a set of tumor-associated antigens in plasma and CaP tissue from patients belonging to the Mayo's prostate SPORC. For this last purpose there is a currently active IRB protocol (Mayo IRB#10-000306). In another project (PCF 2011 Creativity Award, P.I.), the prognostic value of hypoxia-controlled transcripts in resected CaP tissues is being tested. For this purpose the immunostaining of hundreds of tissue slides from a large case-control study was performed at Mayo's TACMA lab. The analysis and interpretation of data is being performed by Drs. George Vassmatzis, John Cheville and Farhad Kosari at Mayo's Center for individualized Medicine. At UMMC, the PI is beginning experiments aimed at validating our findings in the context of the health disparities issue. For this purpose, transfer of materials and data between his lab at UMMC and others throughout Mayo Clinic is critical. This appointment will be beneficial both for the discovery of novel tumor biomarkers and for the development of better therapy for CaP.

Active collaboration was established with Drs. Charles Pound M.D. Head of the Urology Department; Dr. J. Jeffrey Karnes current Director for the Mayo Clinic Department of Urology Radical Prostatectomy/Prostate Cancer Database; and Dr. Srinivasan Vijayakumar M.D., Chair of the Radiation Oncology Department at UMMC. This effort is focused on developing a retrospective research database for patients who were treated by radical prostatectomy in the Department of Urology at UMMC. This project will allow testing the value of potential TAAs and prognostic biomarkers for other populations, particularly those at higher risk. The uniform accumulation of clinical, pathological, and follow-up data on African American patients with operable cancer—with or without adjuvant treatment—will allow estimation of morbidity and

mortality associated with prostatectomy, estimation of long-term outcomes, and assessment of predictors of outcome.

Employment applied

In September 2012 the PI accepted a tenure-track Associate Professor position at the Department of Pathology, University of Mississippi Cancer Center, Jackson, MS (see appended welcoming letter from Dr. James Keeton, Vice Chancellor and Dean) A seeding/Start-up Grant with an estimated performance period of 5 years (09/26/11 – 09/26/16) was received. Level of funding: \$375,000 in direct costs. These funds cover lab equipment, supplies and personnel salaries. Added to external, peer-reviewed funding held by the PI from the DOD (PCRP W81XWH-10-1-0225) and the PCF (2011 Creativity Award) make a total close to \$1,000,000 of combined independent research funding. The general research interests focus of the lab is on modulating immunity to offset the effects of disease and aging. The overall goal is to develop basic research with immediate translational potential and technological applicability in cellular immunotherapy of cancer, immune reconstitution, and regenerative medicine. Ongoing efforts are to develop more effective cancer immunotherapy, particularly immunotherapy of CaP. For more information please visit the following web site: http://cancerinstitute.umc.edu/profiles/gomez_christian.html

Conclusion

We are studying the ways to optimize the effectiveness of therapeutic whole-cell CaP vaccines by tumor-associated hypoxia as a relevant determinant of tumor antigenicity. Our results suggest the value of selected candidates (i.e. HSP60 and hnRNP L) as potential TAAs and immunotherapeutic targets. Performance of their diagnostic value in a validation independent group will establish the clinical utility for early diagnosis of CaP. Additionally, analysis of the overexpression in tumors will help to define the role of these proteins in tumor growth and progression.

So what: The role of pO_2 in tumor biology has been underappreciated. Recently, tumor-associated hypoxia has been associated with malignant progression, metastasis, resistance to therapy, and poor clinical outcome. Our results are contributing to define the potential of hypoxia as a tool in the development of cellular vaccines for CaP and introduce novel diagnostic and prognostic tools not only for CaP, but also for other forms of cancer.

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Appendices

FW: 7th ISABS Conference info - abstract accepted for poster presentation

Page 1 of 2

Gomez, Christian R., Ph.D.

From: Vuk-Pavlovic, Stanimir, Ph.D.
Sent: Monday, May 02, 2011 9:51 AM
To: Gomez, Christian R., Ph.D.
Subject: FW: 7th ISABS Conference info - abstract accepted for poster presentation
FYI, V.

----- Forwarded Message

From: International Society for Applied Biological Sciences - ISABS <info@isabs.hr>
Date: Fri, 29 Apr 2011 18:09:40 +0200
To: "Vuk-Pavlovic, Stanimir, Ph.D." <vuk_pavlovic@mayo.edu>
Subject: 7th ISABS Conference info - abstract accepted for poster presentation



Dear Prof. Stanimir Vuk-Pavlović,

On behalf of the Program Committee of the "**7th ISABS Conference in Forensic, Anthropologic and Medical Genetics and Mayo Clinic Lectures in Translational Medicine**", June 20-24, 2011, Bluesun hotel ELAPHUSA, Bol, Island of Brač, Croatia, we are pleased to inform you that the abstract entitled:

HYPOXIC CELL CULTURE FOR MORE EFFECTIVE CANCER VACCINES

(Control No. ABS-225-ISABS-2011)

has been accepted for Poster Presentation.

Common instructions for poster content and preparation must be followed by the author(s) who have, nevertheless, complete freedom to choose the way they display text, photographs, figures, tables...

The **usable surface** on the poster board will be **100 cm width x 200 cm height**. Adhesive material will be provided.

The presenting author must be near his poster(s) for presentation and discussion at the time indicated in the Conference Program <http://www.isabs.hr/index.php?option=com_content&task=view&id=125&Itemid=154>. Poster presentation numbers will be available in book of abstracts.

Poster mounting will be possible on: **Sunday, June 19, 2011, from 18:00 – 20:00 hrs** and **Monday, June 20, 2011, from 8:00 – 11:00 hrs.**

Removal will be possible on: **Friday, June 24, 2011, from 12:30 hrs – 14:00 hrs.**

Please note that posters not removed until then will be taken down by the staff of the conference centre and will not be stored or sent to the authors after the meeting.

The **poster exhibition** will be accessible for viewing from **Monday, June 20, 2011 (12:00 hrs)** to **Friday, June 24, 2011 (12:30 hrs).**

If you'll need additional information please contact VenEvent, our official conference service agency at isabs2011@venevent.com [<mailto:isabs2011@venevent.com>](mailto:isabs2011@venevent.com).

We look forward to welcoming you in Bol in June.
With kind regards,

Vedrana Skaro, Ph.D.
General Secretary

Organizing Committee of the Conference



International Society for Applied Biological Sciences

Domobranska 21, 10000 Zagreb, Croatia
info@isabs.hr • www.isabs.hr

----- End of Forwarded Message

6/6/2011

PROGNOSTIC VALUE OF HYPOXIA-CONTROLLED GENES IN HIGH-RISK PROSTATE CANCER

Christian R. Gomez, Jan Marie Munz, Farhad Kosari, R. Jeffrey Karnes, John C. Cheville, Cristiane M. Ida, Thomas J. Sebo, Asha A. Nair, Hui Tang, George Vasmatazis, Stanimir Vuk-Pavlović. Mayo Clinic, Rochester, MN, USA.

Tumor-associated hypoxia has been associated with malignant progression, metastasis, resistance to therapy, and poor clinical outcome in prostate cancer (CaP). Prompted by the evidence that hypoxia affects CaP, we studied global gene expression in 100 CaP tissues and 71 samples of adjacent benign tissues. RNA was extracted from cancer cells isolated by laser-capture microdissection (LCM) or without isolation ("bulk tissue"). We found 24 hypoxia-associated genes significantly overexpressed in CaP ($p \leq 0.02$), both in bulk tissue and LCM. Among hypoxia-associated genes, the disc large (drosophila) homolog-associated protein 5 (DLG7), hyaluronan-mediated motility receptor (HMMR) and cyclin B1 (CCNB1) were associated with Gleason score and systemic progression. Since the products of HMMR and CCNB1 have been recently identified as molecular markers of CaP progression, we postulated that DLG7 has prognostic value also. To test this hypothesis we measured transcript levels for DLG7 in a 150-pair case-control cohort. The cases (progression to systemic disease within five years of surgery) and controls (no progression within seven years) were matched for clinical and pathologic prognostic variables, including grade, stage, and preoperative serum levels of PSA. The overall prognostic ability of DLG7, as tested in receiver operating characteristic analysis was of 0.74 (95% CL, 0.68 to 0.8), independent of the for ERG status. Overall, our data indicate that DLG7, a hypoxia-controlled gene have a prognostic value in high-risk CaP. Introduction of oxygen tension as a variable may constitute a tool for the identification of novel biomarkers for CaP.

Support: PCF Creativity Award (CRG), DOD PC094680 (CRG), Minnesota Partnership for Biotechnology and Medical Genomics (GV), Mayo Clinic Prostate SPORC 5P50CA091956 (FK, SV-P), Adelyn Luther, Singer Island, Florida (SV-P); and Mayo Clinic Cancer Center (SV-P).



Control/Tracking Number: 11-A-775-AACR
 Activity: Abstract Submission
 Current Date/Time: 9/12/2011 11:07:59 AM

Targeting hypoxia for more effective immunotherapy of prostate cancer

Short Title:
 hypoxia and immunotherapy of CaP

Author Block: Christian R. Gomez¹, Claire A. Schreiber², Gaylord J. Knutson², Cristine Charlesworth², Stanimir Vuk-Pavlovic². ¹University of Mississippi Medical Center, Jackson, MS; ²Mayo Clinic, Rochester, MN

Abstract:

In early clinical trials, vaccination with allogeneic cancer cells enhanced the overall survival in some patients with malignancies. Because the administered vaccine cells were cultured at $pO_2=20$ kPa, it is unclear whether they provided an adequate antigen match to tumor cells *in situ* where pO_2 is generally much lower. Thus, we postulate that hypoxically grown vaccine cells will provide a better antigen match to tumors *in situ*. We are testing this hypothesis by studying the effects of hypoxia on prostate cancer (CaP) cells. LnCaP and VCaP cells were cultured at $pO_2=2$ kPa or 20 kPa and analyzed by 2-D gel electrophoresis. The difference in pO_2 affected the proteome mostly quantitatively (*i.e.*, by change in spot intensity). Using a threshold of fivefold change, we found that hypoxia decreased the levels of thirteen proteins and increased of four in VCaP cells. These results are in line with the reports showing that hypoxia affects expression only of a small fraction of total cellular protein and that the content of total protein is not altered significantly. To determine if the hypoxia-dependent changes affected immune reactivity of the cells, we took advantage of the spontaneous autoantibodies against tumor-associated antigens (TAAs). Hence, we compared the binding to lysates of LnCaP and VCaP cells of antibodies pooled from CaP patients ($n=25$), healthy controls ($n=25$), rheumatoid arthritis ($n=17$), colorectal cancer ($n=10$) and lung cancer ($n=10$). CaP patient sera plasma reacted with numerous spots, some even in control groups and thus considered nonspecific. CaP sera plasma specifically bound to seven spots; four were hypoxia-specific. All selected spots were excised from the gel, trypsin-digested, and identified by MALDI-TOF mass spectrometry as heat shock 70 kDa protein 4; protein disulfide isomerase A3; heterogeneous nuclear ribonucleoprotein L and leucine-rich repeat-containing protein 47. With the exception of the latter, the proteins have been identified or validated as TAAs before, but apparently none in CaP. CaP patient antibodies bound to lysates of LnCaP cells grown at $pO_2=2$ kPa, but less than to such VCaP cell lysates. Our results confirmed the findings that hypoxia affects the proteome mostly quantitatively. Lysates of hypoxic CaP cells revealed novel potential TAAs suggesting the potential immunologic relevance of antigens in the hypoxic proteome. Thus, pO_2 control can provide a tool for the development of more effective immunotherapy for CaP and possibly other cancers.

Present address CRG: University of Mississippi Cancer Institute, Jackson, MS, USA. Support: DOD PC094680 (CRG), PCF Creativity Award (CRG), Mayo Clinic Prostate SPORC 5P50CA091956 (SV-P); Mrs. Adelyn L. Luther, Singer Island, Florida and Mayo Clinic Cancer Center (SV-P).

Category (Complete): Tumor immunology targets

Keywords/Indexing (Complete): Tumor hypoxia ; Immunotherapy ; Prostate cancer ; Tumor antigen

Chemical Structure Disclosure (Complete):

Choose Chemical Structure Disclosure: NOT APPLICABLE. No compounds with defined chemical structures were used.
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(220319_1) - Prognostic value of hypoxia-associated genes in prostate cancer

Christian R. Gomez¹, Farhad Kosari², Jan Marie Munz², Claire Schreiber², Gaylord Knutson², Cristine Charlesworth², R. Jeffrey Karnes², John Cheville², George Vasmatazis², Stanimir Vuk-Pavlovic².

¹University of Mississippi Medical Center, Jackson, MS, ²Mayo Clinic, Rochester, MN.

Tumor-associated hypoxia has been associated with malignant progression, metastasis and resistance to therapy. We studied the role of oxygen tension (pO₂) in modulating the properties of cultured human prostate cancer (CaP) cells. In air, where oxygen tension (pO₂) is approximately 20 kPa, cultured CaP cells expressed lower levels of transcripts associated with cancer and urologic disease; reducing pO₂ to 2 kPa made these levels comparable to those in surgical CaP specimens suggesting that hypoxically cultured cells, rather than normoxically cultured cells, express genes at levels akin to those in tumor cells in situ. In human primary CaP tissue, transcripts of hypoxia-associated genes DLG7, CCNB1 and HMMR were overexpressed and associated with Gleason score and disease prognosis; this implies the potential prognostic value of these transcripts. While hypoxia affected the CaP cell proteome mostly quantitatively, it did affect expression of tumor-associated antigens and suggested the potential therapeutic relevance of the hypoxic antigenic landscape. Overall, our results suggest that hypoxia modifies cellular properties of cultured CaP cells towards a phenotype more similar to tumor cells in situ. Introduction of pO₂ as a variable can constitute a tool for the identification of more effective prognostic biomarkers and development of better therapy for CaP and, possibly, other tumors.

Category: Prognostic Signatures

Keywords: hypoxia; biomarkers; prognosis

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Activity: Abstract Submission

Current Date/Time: 10/24/2011 1:26:37 PM

Targeting hypoxia for the identification of novel biomarkers for prostate cancer

Short Title:

Hypoxia biomarkers prostate cancer

Author Block: Christian R. Gomez¹, Jan Marie Munz², Farhad Kosari², Jeffrey Karnes², John Cheville², Cristiane Ida², Thomas Sebo², George Vasmatzis², Stanimir Yek-Pavlovic². ¹University of Mississippi Medical Center, Jackson, MS; ²Mayo Clinic, Rochester, MN

Abstract:

Tumor-associated hypoxia has been associated with malignant progression, metastasis, resistance to therapy, and poor clinical outcome in prostate cancer (CaP). Prompted by the evidence that hypoxia affects CaP, we studied global gene expression in 100 CaP tissues and 71 samples of adjacent benign tissues. RNA was extracted from cancer cells isolated by laser-capture microdissection (LCM) or without isolation ("bulk tissue"). We found 24 hypoxia-associated genes significantly overexpressed in CaP ($p \leq 0.02$), both in bulk tissue and LCM. Among hypoxia-associated genes, the disc large (drosophila) homolog-associated protein 5 (DLG7), hyaluronan-mediated motility receptor (HMMR) and cyclin B1 (CCNB1) were associated with Gleason score and systemic progression. Since the products of HMMR and CCNB1 have been recently identified as molecular markers of CaP progression, we postulated that DLG7 has prognostic value also. To test this hypothesis we measured transcript levels for DLG7 in a 150-pair case-control cohort. The cases (progression to systemic disease within five years of surgery) and controls (no progression within seven years) were matched for clinical and pathologic prognostic variables, including grade, stage, and preoperative serum levels of PSA. The overall prognostic ability of DLG7, as tested in receiver operating characteristic analysis was of 0.74 (95% CL, 0.68 to 0.8), independent of the for ERG status. Overall, our data indicate that DLG7, a hypoxia-controlled gene have a prognostic value in high-risk CaP. To better define the value of DLG7 as a molecular predictor of CaP progression, we are currently assessing DLG7 protein levels by immunohistochemistry. Then we will analyze the association of DLG7 levels with clinical parameters. Introduction of oxygen tension as a variable may constitute a tool for the identification of novel biomarkers for CaP.

Present address CRG: University of Mississippi Cancer Institute, Jackson, MS, USA. Support: DOD PC094680 (CRG), PCF Creativity Award (CRG), Mayo Clinic Prostate SPOR 5P50CA091956 (SV-P); Mrs. Adelyn L. Luther, Singer Island, Florida and Mayo Clinic Cancer Center (SV-P).

Author Disclosure Information: C.R. Gomez: None. J. Munz: None. F. Kosari: None. J. Karnes: None. J. Cheville: None. C. Ida: None. T. Sebo: None. G. Vasmatzis: None. S. Yek-Pavlovic: None.

Sponsor (Complete):

Category and Subclass (Complete): MCB03-01 Molecular marker studies

Keywords/Indexing (Complete): Hypoxia ; Biomarker ; Prostate cancer

Research Type (Complete): Translational research

Submission Details (Complete):

*Primary Organ Site: Genitourinary cancers: prostate

*Special Consideration: Not Applicable

*Choose Chemical Structure Disclosure: NOT APPLICABLE. No compounds with defined chemical structures were used.

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November 22, 2011

SENT BY FEDERAL EXPRESS

Christian R. Gomez, Ph.D.
1840 Devine Street
Jackson, MS 39216

Dear Dr. Gomez:

I am pleased to offer you an appointment at Mayo Clinic as a research collaborator with Dr. Stanimir Vuk-Pavlovic, in the Division of Hematology for twelve months.

In keeping with research guidelines, the collaborator appointment is limited to no more than 90 days on campus during your appointment year. This appointment does not include any salary, honorarium, or benefits from Mayo Clinic.

As a research collaborator, you will be required to comply with Mayo Clinic policies during this appointment. You will not be eligible for patient or research subject contact, including clinical or surgical observation, remote access to the electronic medical record, or any other clinical applications.

Please arrange for health care insurance coverage for any hospitalization or physician services that may be required while at Mayo Clinic.

It is required that you read and complete the following forms and fax to (507) 538-0786 or e-mail as scanned documents to researchtp@mayo.edu:

- Appointment Information
- Confidential Information
- Intellectual Property
- Mayo Health Insurance Portability and Accountability (HIPAA) training
- Mayo Clinic Integrity Program - Patient Privacy & Security

Dr. Christian Gomez

2

November 22, 2011

If you have questions concerning your appointment, please let me know. I can be reached at golla.alice@mayo.edu or telephone at (507) 284-3281.

Sincerely,

A handwritten signature in blue ink that reads "Alice".

Alice Golla, Staffing Specialist
Human Resources

AG:ljb

Enclosures

cc: Dr. Stanimir Vuk-Pavlovic



Office of the Vice Chancellor
for Health Affairs
2500 North State Street
Jackson, Mississippi 39216-4505
(601) 984-1010

September 28, 2011

Dr. Christian Gomez
Department of Pathology/Cancer Institute
UMMC -- Campus Mail

Dear Dr. Gomez:

On behalf of the University of Mississippi Medical Center (UMMC), I want to personally congratulate you on your appointment as Associate Professor in the Department of Pathology in the School of Medicine.

I am certain your talents will enrich this department and contribute to the exciting direction in which our institution is moving.

Best wishes and I look forward to working with you in the future.

Sincerely,

James E. Keeton, M.D.
Vice Chancellor and Dean

JEK/pr

July 27, 2012

CURRICULUM VITAE

Name: Christian René Gomez Basaure Ph.D.

Birthdate:

Marital Status:

Spouse:

Children:

Home Address:

E-mail: crgomez@umc.edu

Web Page: http://cancerinstitute.umc.edu/profiles/gomez_christian.html

Present Position: Associate Professor, Department of Pathology
Associate Professor, Department of Radiation Oncology (pending)
Full member Cancer Institute
University of Mississippi Medical Center
2500 N. State St. Suite G657, Jackson, MS 39216
Office: 601-815-3060, Fax 601-815-6806

Education

1988 - 1995 B.S. and M.S. in Biochemistry, School of Chemical and Pharmaceutical Sciences,
University of Chile

1997-2003 Ph.D. Biomedical Sciences, University of Chile, Faculty of Medicine Date of Ph.D.
completion January 2004

Research Training

1992 - 1994 Undergraduate thesis: Involvement of the Sodium/ATPase pump in chronic renal failure,
Advisor: Dr. Miriam Alvo, Department of Physiology, University of Chile School of
Medicine, Santiago, Chile

1995 - 1997 Research assistant: Glucocorticoid receptors in the development of Rheumatoid Arthritis:
Development of a rat model, Advisor: Dr. Annelise Goecke, Department of
Physiology, University of Chile School of Medicine, Santiago, Chile

1998 - 2000 Research assistant: CAAT/enhancer-binding protein signaling during the acute phase
response of aged Fisher 344 rats, Advisor: Dr. Robin Walter, Department of Cellular and
Molecular Biology, University of Chile School of Medicine, Santiago, Chile

2000 - 2004 Doctoral dissertation: Macrophage inflammatory protein 1-alpha as a modulating factor of
the acute phase response: extension to the inflammatory response in aged individuals,

Advisor: Dr. Felipe Sierra, Department of Cellular and Molecular Biology, University of Chile School of Medicine, Santiago, Chile

2004 - 2008 Postdoctoral Fellow: Aging and inflammatory responses. Supervisor: Dr. Elizabeth J. Kovacs, Loyola University Chicago, Stritch School of Medicine, Department of Cell Biology, Neurobiology and Anatomy and Department of Surgery, Maywood, IL

International Courses

Natural Antibodies in the Maintenance of Tolerance to Self: Lessons from Physiology and Therapy, Program of Immunology, Faculty of Medicine, University of Chile, Santiago, Chile, 15-16 December 1998

International Symposium and Training Course: "Cellular Signaling From Plasma membrane to the Nucleus", Program of Cellular and Molecular Biology, Faculty of Medicine, University of Chile, Santiago, Chile, 12-23 July 1999

International Symposium and Training Course: "International Course on Techniques for the Study of Functional Genomics", Program of Cellular and Molecular Biology, Faculty of Medicine, University of Chile, Santiago, Chile, 19 June – 1 July 2000

Training Course: "Molecular Biology of Aging", Marine Biological Laboratory, Woods Hole, MA, USA, 31 July – 19 August 2006

Faculty Appointments

2004 - 2007 Research Associate, Loyola University Chicago, Stritch School of Medicine, Department of Cell Biology, Neurobiology and Anatomy, Maywood, IL

2007 - 2008 Research Associate, Loyola University Chicago, Stritch School of Medicine, Department of Surgery, Maywood, IL

2008 - 2011 Research Associate, Stem Cell Laboratory, Department of Oncology, Mayo Clinic Cancer Center, Rochester, MN

2009 - 2011 Assistant Professor of Biochemistry/Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN

2010 - 2011 Assistant Professor, Division of Preventive, Occupational, And Aerospace Medicine, Mayo Clinic, Rochester, MN

2011 - Date Associate Professor, Department of Pathology, University of Mississippi Medical Center, Jackson, MS

2011 - Date Full Member, University of Mississippi Cancer Center, University of Mississippi Medical Center, Jackson, MS

2011 - Date Research Collaborator, Mayo Clinic, Rochester, MN

2012 - Date Full Member, The School of Graduate Studies' Graduate Faculty, University of Mississippi Medical Center, Jackson, MS

2012 - Date Associate Professor, Department of Radiation Oncology, University of Mississippi Medical Center, Jackson, MS (pending)

Professional Awards

1998-2002 Chilean National Council for Science and Technology (CONICYT) doctoral scholarship

2000 International Travel Award: "Identification of genes that are differentially expressed during the acute phase response of senescent animals", Lab. Dr. Christian Cell, Lankenau Institute for Medical Research, Thomas Jefferson University, Wynnewood, PA, USA

2002 International Travel Award: "Characterization of differential hepatic expression of the chemokines MIP-1 α , in aged rats, injected with bacterial endotoxin (LPS)", Lab. Dr. Christian Sell and Lab. Dr. Vincent Cristofalo, Lankenau Institute for Medical Research, Thomas Jefferson University, Wynnewood, PA, USA

2002	International Travel Award: "Standardization of the measurement of tissue and circulating levels of cytokines during the acute phase response of aged rats", Lab. Dr. Elizabeth J. Kovacs, Stritch School of Medicine, Loyola University, Maywood, IL, USA
2001	Distinguish Award for the "Best dissertation project on Gerontological Studies" Interdisciplinary Program for Gerontological Studies, University of Chile, Santiago, Chile
2004	Travel award to attend the Annual Meeting of the Society for Leukocyte Biology, Toronto, Canada
2004	Doctoral Medal, University of Chile, Santiago, Chile
2005	Travel award to attend the Annual Meeting of the Society for Leukocyte Biology, Oxford, England
2006	Young Investigator Travel Award to attend the Twenty-Ninth Annual Conference on Shock, Broomfield, CO, USA
2006	Travel award to attend the Annual Meeting of the Society for Leukocyte Biology, San Antonio, TX, USA
2010	AACR Minority Scholar in Cancer Research Award to attend the AACR Special Conference, Tumor Immunology: Basic and Clinical Advances. Miami, FL
2011	Prostate Cancer Foundation. Treatment Sciences Creativity Awards 2011

Professional Society Membership and Activities

2004 - 2008	Society for Leukocyte Biology, Member
2004 - 2008	Shock Society, Member
2009 - Date	American Association for Cancer Research (AACR), Associate Member
2009 - Date	Mayo Clinic Alumni Association, Member
2009 - Date	AACR, Minority Scholar in Cancer Research, Member

Media Related Quotes and Interviews

2005	"Healthy Aging", Interview, University of Santiago Radio. Santiago, Chile
------	---

Journal Review Activity

2010 - Date	Ad Hoc Reviewer: Journal of Leukocyte Biology, American Journal of Physiology-Advances in Medical Education
2012 - Date	Editorial Board of Conference Papers in Immunology

Grant Review Panels

2012 - Date	Training Clinical and Experimental Therapeutics peer review panel of the 2012 Prostate Cancer Research Program for the Department of Defense Congressionally Directed Medical Research Programs.
-------------	--

Teaching Experience

1999, 2001	Teaching Assistant, Course of Cellular Biology for Kinesics therapy and Occupational therapy and Medical Technology (First year students), Faculty of Medicine, University of Chile, Santiago, Chile
2002-2003	Teaching Assistant, Seminars on Biotechnology for Medical technology students (second year students), Mention clinical bio-analysis, Hematology and Blood bank, Faculty of Medicine, University of Chile, Santiago, Chile
2002-2003	Teaching Assistant, Cellular Biology course for Biochemistry students (fourth year students), Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile

2003-2004	Teaching Assistant, Workshop for Integration of Basic Sciences for Medical students (first year students), Faculty of Health Sciences, University Diego Portales, Santiago, Chile
2003-2006	Teaching Assistant, Course Structure and Function I for Medical students (first year students), Faculty of Health Sciences, University Diego Portales, Santiago, Chile
2003	Teaching Assistant, Course of Cell Biology for Ph.D. students, Faculty of Medicine, University of Chile, Santiago, Chile
2003	Teaching Assistant, Course of Advanced Genetics for Ph.D. students, Faculty of Medicine, University of Chile, Santiago, Chile
2004	Teaching Assistant, course of Cell Biology for Nursery and Medical Technology students (first year students), Faculty of Health Sciences, University Diego Portales, Santiago, Chile
2004	Teaching Assistant, Seminars in Molecular Biology for Medical Technology (fourth year students), Mention clinical Bio-analysis, Hematology and Blood bank, Faculty of Medicine, University of Chile, Santiago, Chile
2006	TEACHING ASSISTANT, MEDICAL HISTOLOGY, THE STRITCH SCHOOL OF MEDICINE, LOYOLA UNIVERSITY MEDICAL CENTER, MAYWOOD, IL

Research Supervision

2005 Co-mentor: Stephanie Hirano, M.D. Student

2005 Mentor: Ying Peng, Ph.D. Candidate
Christine Regnell, M.S. Candidate
Shirin Birjandi, Ph.D. Candidate

All the students were at Elizabeth J. Kovacs' Laboratory at The Burn and Shock Trauma Institute, Loyola University Medical Center, Maywood, IL

2006-8	Mentor: Freddy Bustos, Constanza Fernández, Ana María Duhalde, M.D. Students, Methodology in research rotation, Universidad Diego Portales, Santiago, Chile
2009	Co-Mentor: Freddy Bustos, M.D. Student, research rotation, Stem Cells Lab, Mayo Clinic Cancer Center, Rochester, MN
2010-Date	Claire A. Schreiber, Luther College, Decorah, IA Research Assistant, Stem Cells Lab, Mayo Clinic Cancer Center, Rochester, MN
2011	Lauren Ulbrich, St. Mary's University, Winona, MN Summer student, Stem Cells Lab, Mayo Clinic Cancer Center, Rochester, MN
2011-Date	Tangeng Ma, Research Scientist III, University of Mississippi, Cancer Institute, Jackson, MS
2011-Date	Elizabeth Tarsi, Research Scientist II, University of Mississippi, Cancer Institute, Jackson, MS
2012-Date	Abdelouahid Elkhattouti, Postdoctoral Fellow I, University of Mississippi, Cancer Institute, Jackson, MS
2012	Appifani Binion, Tougaloo College, Tougaloo, MS, summer student, University of Mississippi, Cancer Institute, Jackson, MS

Research Grant Support

Ongoing:

Title: Enhancing therapeutic cellular prostate cancer vaccines (PC094680) (P.I.).

Time commitment: 50%

Supporting agency: Department of Defense. New Investigator Award

Performance period: 04/15/10 – 03/31/12

Level of funding (direct costs): \$225,000

Project goals: The overall goal of the proposed studies is designed to optimize the effectiveness of therapeutic whole-cell CaP vaccines. We hypothesize that hypoxically cultured CaP cells are more similar in their antigen landscape to CaP cells in situ than are normoxically cultured CaP cells

Specific aims: 1) To identify oxygen-tension responsive genes and proteins in the cells comprising a clinical-grade CaP cellular vaccine. 2) To validate differentially expressed molecules in CaP tissue in association with tissue hypoxia

Key personnel receiving salary support from this project: 100% effort CR Gomez. 50% effort allied staff

Title: Hypoxia-regulated DLG7 in prostate cancer carcinogenesis and prognosis. (P.I.)

Time commitment: proposed 50%

Supporting agency: Prostate Cancer Foundation. Treatment Sciences Creativity Awards 2011 Performance period: 05/01/11 – 05/01/13

Level of funding: \$300,000 (direct costs)

Project goals: We found the transcripts of the discs large homolog-associated protein 5 (DLG7), a hypoxia-regulated gene, overexpressed in human primary prostate cancer and human prostate cancer cell lines. The overall goal of the proposed studies is to validate the role of DLG7 role in tumor progression

Specific aims: 1) To measure the levels of DLG7 transcripts in resected CaP tissues and study the association with survival. 2) To overexpress DLG7 in prostate cells (normal epithelium and tumorigenic cells) and compare tumorigenesis in the context of hypoxia.

Key personnel receiving salary support from this project: 50% effort CR Gomez. 100% effort postdoctoral fellow

Title: Improving effectiveness of cancer immunotherapy of prostate cancer. (P.I.)

Supporting Institution: University of Mississippi Cancer Institute–Seeding/Start-up Grant, Jackson, MS

Performance period: 09/26/11 – 09/26/16

Project goals: To characterize the antigenic landscape of hypoxically cultured prostate cancers cells and compare it to that of normoxic CaP cells. Testing of the role of the hypoxia-controlled gene–DLG7–in tumor progression

Key personnel receiving salary support from this project: 100% effort technical personnel and one postdoctoral fellow

Completed:

Title: Hyperbaric oxygen as mobilizer of stem cells and progenitors in senescent mice (Stanimir Vuk-Pavlovic, P.I.). Co P.I.

Time commitment: 30%

Supporting agency: Mayo Clinic, Division of Preventive, Occupational and Aerospace Medicine Small Grant Awards

Performance period: 04/01/09 – 12/30/09

Level of funding: \$17,500

Project goals: The effects of hyperbaric oxygen (HBO) on mobilization of hematopoietic and stem and progenitor cells (HSPCs) and mesenchymal stromal cells (MSCs) from bone marrow into circulation of old mice were explored.

Specific Aims: 1) To measure the effects of HBO in young and old mice by flow cytometry after labeling white blood cells with pertinent fluorescent immunoreagents for HSPCs and MSCs. 2) To measure the levels of selected circulating cytokines involved in HSPCs and MSCs mobilization.

Key personnel receiving salary support from this project: 3% effort CR Gomez

Title: Hyperbaric oxygen as mobilizer of stem cells and progenitors in senescent mice. Extension of funds for 2010 (Stanimir Vuk-Pavlovic, P.I.). Co P.I.

Time commitment: 30%

Supporting agency: Mayo Clinic, Division of Preventive, Occupational and Aerospace Medicine Small Grant Awards

Performance period: 04/01/10 – 12/31/10

Level of funding: \$10,000

Project goals: The mechanisms of age-related impairment of mobilization of both hematopoietic stem and progenitor cells (HSPCs) and mesenchymal stromal cells (MSCs) from bone marrow by hyperbaric oxygen (HBO) into circulation of old mice were studied. Specific Aims: 1) To analyze the effects of aging and HBO on the expression of SDF-1/CXCR4 system, the critical regulator of SPCs function and homing. 2) To analyze the effects of aging and HBO on the regulation of nitric oxide (\bullet NO)–mediated mechanism of MSCs mobilization by HBO synthesis.

Key personnel receiving salary support from this project: 3% effort CR Gomez

Applied

Title: Hypoxia enhances prostate cancer radioresistance by promoting cancer cell stemness. P.I.

Funding agency: BD Biosciences Research Grant Program-Stem Cell Grant.

Time commitment: 5%. Performance period: 07/01/12 – 07/01/14. Level of funding: \$10,000.

Project goal: test the hypothesis that a low oxygen environment (where radiation is less effective due to the lack of the oxygen effect) increases the fraction of cancer stem cells in the population giving thus rise to a more resistant tumor.

Key personnel receiving salary support from this project: 10% effort flow cytometry core personnel.

Title: A gene panel predictive of outcome in men at high-risk prostate cancer: Prognostic performance in African Americans

Funding agency: Bayer-Grants4Targets

Time commitment: 5%. Performance period: 06/01/12 – 06/01/14.

Level of funding: \$150,000.

Project goal: test the hypothesis that a panel of hypoxia-sensitive genes and TAAs identified in Caucasians has prognostic value in patients from other races with diagnosed CaP and in other cancers.

Key personnel receiving salary support from this project: 100% effort postdoctoral fellow.

Planned:

“Hypoxia and tumor microenvironment to improve cell immunotherapy for prostate cancer”. NIH, R01. \$250,000/\$1,250,000 (direct costs). Period: 07/01/12 – 06/30/16. P.I.

Research Interests

- Immunosenescence: The effects of advanced age on inflammation, immunoregulation, and injury. Age-dependent defects in cytokine networks. Interleukin-6: age-related effects on cellular and systemic immune responses. Hormone replacement and cell mediated immunity in the aged.
- Immunotherapy for prostate cancer: Small molecules as modulators of the tumor microenvironment. Strategies aimed at improving delivery of whole-cell cancer vaccines by improving their antigenicity.
- Restoring immunity in the aged: Hyperbaric oxygen therapy as mobilizer of stem cells and progenitors in senescent individuals.

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Manuscripts in Preparation

1. **Gomez, C.R.**, Knutson, G., Schreiber C.A., Vuk-Pavlovic S., Low oxygen tension as a tool for the development of better prostate cancer cell vaccines
2. **Gomez, C.R.**, Kosari, F., Munz, J.M., Schreiber, C., Knutson, G., Charlesworth, C., Karnes, R. J., Cheville, J., Vasmatazis, G., Vuk-Pavlovic, S. Prognostic value of hypoxia-associated genes in prostate cancer
3. Elkhattouti, A., **Gomez, C.R.** Aging Niche in Senescence-Related Pathology (submitted).

Research Reports and Abstracts

1. **Gomez, C.R.**, Walter, R. and Sierra F. 2001. Regulation of gene expression of cytokines during aging. Symposium, "Oxidative stress, cellular responses and modulator factors", XXIV annual meeting of the Chilean Society of Biochemistry and Molecular Biology. Chillán, Chile, p.23
2. **Gomez, C.R.**, Walter, R. and Sierra, F. 2002. Expression of MIP-1 α during the acute phase response of aged rats. XVI annual meeting of the Chilean Society of Cellular Biology. Puerto Varas, Chile, p. 22
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30. Nomellini, V., **Gomez, C.R.**, and Kovacs E.J. 2007. Pulmonary neutrophil accumulation after burn injury is exacerbated with age. 30th Annual Conference on Shock. Baltimore, MA, USA
31. Bustos, F., Fernández, C., Duhalde, A., **Gomez, C.R.** and Salinas, D.G. 2007. Motivational profile of the students that enter and remain in a Medical School, University Diego Portales: A retrospective study. International IV Convention of Medical Education. Medical School, Catholic University. Santiago, Chile
32. **Gomez, C.R.**, Karavitis, J., Ramirez, L., Nomellini, V., and Kovacs E.J. 2007. Interleukin-6 contributes to age-related alteration of cytokine production in macrophages. 40th Annual Meeting of the Society for Leukocyte Biology. Cambridge, MA, USA
33. Nomellini, V., **Gomez, C.R.**, and Kovacs E.J. 2007. Decreased tight junction formation in lungs of aged mice following injury. 40th Annual Meeting of the Society for Leukocyte Biology. Cambridge, MA, USA

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37. Kovacs, E.J., **Gomez C.R.**, and Nomellini, V. 2008. Aging and injury: aberrant inflammatory responses and therapeutic interventions. US-Japan Cooperative Medical Sciences Program. Immunosenescence Workshop, San Francisco, CA
38. Nomellini, V., **Gomez, C.R.**, and Kovacs, E.J. 2008. Aberrant neutrophil sequestration in the lungs of aged mice after burn injury is paralleled by a decrease in the neutrophil chemokine receptor, CXCR2. 41th Annual Meeting of the Society for Leukocyte Biology. Denver, CO
39. **Gomez, C.R.**, Knutson, G., G.J., Vuk-Pavlović, S. 2008. Oxygen–tension Dependent Proteome Modulation in Human Cancer Cells. Symposium: Hypoxia, Ischemia, and Inflammation: Essential Connections. Boston, MA
40. **Gomez, C.R.**, Kosari, F., Schreiber, C.A., Knutson G.J., Vasmatazsis, G., Vuk-Pavlović S. 2010. Hypoxic exposure as a novel strategy to identify tumor-associated antigens in prostate cancer. Tumor Immunology: Basic and Clinical Advances. Miami Beach, FL
41. **Gomez, C.R.**, Knutson G.J., Schreiber, C.A., Kosari, F., Vasmatazsis, G., Vuk-Pavlović S. 2011. Hypoxia affects gene expression and proteome of prostate cancer cells. 102th AACR Annual Meeting. Orlando, FL
42. **Gomez, C.R.**, Knutson G.J., Schreiber, C.A., Vuk-Pavlović S. 2011. Age–dependent mobilization of mesenchymal stromal cells by hyperbaric oxygen. 17th International Society for Cellular Therapy Meeting. Rotterdam, The Netherlands
43. **Gomez, C.R.**, Kosari, F., Schreiber, C.A., Knutson G.J., Vasmatazsis, G., Vuk-Pavlović S. 2011. Hypoxic cell culture for more effective cancer vaccines. International Society for Applied Biological Sciences Conference on Forensic Genetics (ISABS). Bluesun hotel ELAPHUSA, Bol, Island Brač, Croatia
44. **Gomez, C.R.**, Munz, J.M., Kosari, F., Karnes R.J., Cheville, J., Ida, C.M., Sebo, T.J., Nair, A.A., Tang, H., Vasmatazsis, G., Vuk-Pavlović S. 2011. Prognostic value of hypoxia-controlled genes in high-risk prostate cancer. Eighteen Annual Prostate Cancer Foundation Scientific Retreat. Incline Village, Lake Tahoe, NV
45. **Gomez, C.R.**, Schreiber, C.A., Knutson G.J., Charlesworth, M.C., Vuk-Pavlović S. 2011. Targeting hypoxia for more effective immunotherapy of prostate cancer. Molecular Targets and Cancer Therapeutics AACR-NCI-EORTC International Conference. San Francisco, CA
46. **Gomez, C.R.**, Kosari, F., Munz, J.M., Schreiber, C., Knutson, G., Charlesworth, C., Karnes, R. J., Cheville, J., Vasmatazsis, G., Vuk-Pavlovic, S. 2012. Prognostic value of hypoxia-associated genes in prostate cancer. Advances in Prostate Cancer Research. Orlando, FL
47. **Gomez, C.R.**, Munz, J.M., Kosari, F., Karnes R.J., Cheville, J., Ida, C.M., Sebo, T.J., Vasmatazsis, G., Vuk-Pavlović S. 2012. Targeting hypoxia for the identification of novel biomarkers for prostate cancer. 103th AACR Annual Meeting. Chicago, IL

Invited Lectures, Seminars, and Presentations

- "cDNA Microarrays Analysis Shows Differential Expression of a Subset of Genes, During the Hepatic Acute Phase Response in Aged Fisher 344 Rats". XLIII Annual Meeting of the Biology Society of Chile. Pucón, Chile, November 2000
- "Hepatic Response to Inflammation during Aging". International Symposium Molecular and cellular basis of Aging. ICBM, Faculty of Medicine, University of Chile. Santiago, Chile, June 2001
- "Technologies of information and communication in a course for integrated teaching of biology". V workshop of Education in Health Sciences. Faculty of Medicine, University of Chile. Santiago, Chile, May 2004
- "Inflammatory responses during Aging: The Good, the Bad and the Ugly", Burn and Shock Trauma Institute, Loyola University Medical Center. Maywood, IL, October 2005
- "Inflammatory responses during Aging: From the bench to the bedside", Veteran Affairs Center of Physical Rehabilitation. La Florida, Chile, November 2005
- "Inflammatory responses during Aging", Veteran Affairs Medical Center, Las Condes, Chile. November 2005
- "Advanced age exacerbates the pulmonary inflammatory response after lipopolysaccharide exposure", Twenty-Ninth Annual Conference on Shock. Broomfield, CO, USA, June 2006
- "Inflammatory responses and Aging", Faculty of Health Sciences, Diego Portales Santiago, Chile. November 2007
- "Inflammatory responses and Aging", Faculty of Health Sciences, Burn and Shock Trauma Institute, Loyola University Medical Center. Maywood, IL, March 2008
- "Translational studies of hyperbaric oxygen effects at Mayo", Division of Preventive, Occupational, And Aerospace Medicine Monthly Research Seminars, Mayo Clinic, Rochester, MN. March 2010
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Age-dependent response of murine female bone marrow cells to hyperbaric oxygen

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Abstract Consequences of age on the effects of hyperbaric oxygen (HBO) on bone marrow (BM) derived stem cells and progenitors (SCPs) are largely unknown. We treated 2- and 18-month old C57BL/6 female mice by HBO. Hematopoietic stem cells and progenitors, enumerated as colony-forming units in culture, were doubled only in peripheral leukocytes and BM cells of young mice receiving HBO. In old

mice colony-forming unit fibroblast numbers, a measure of mesenchymal stromal cells (MSCs) from BM, were high but unaffected by HBO. To further explore this finding, in BM-MSCs we quantified the transcripts of adipocyte early-differentiation genes peroxisome proliferator-activated receptor- γ , CCAAT/enhancer binding protein- β and fatty-acid binding protein 4; these transcripts were not affected by age or HBO. However, osteoblast gene transcripts runt-related transcription factor 2, osterix (OSX) and alkaline phosphatase (AP) were twofold to 20-fold more abundant in MSCs from old control mice relative to those of young control mice. HBO affected expression of osteoblast markers only in old MSCs (OSX gene expression was reduced by twofold and AP expression was increased threefold). Our data demonstrate the impact of aging on the response of BM SCPs to HBO and indicate the potentially different age-related benefit of HBO in wound healing and tissue remodeling.

Keywords Aging · Hyperbaric oxygen · Hematopoietic progenitor cells · Mesenchymal stromal cells

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Abbreviations

AP	Alkaline phosphatase
aP2	Fatty-acid binding protein 4
BM	Bone marrow
BMT	Bone marrow transplant
C/EBP- β	CCAAT/enhancer binding protein- β

CFU-C	Colony-forming units in culture
CFU-F	Colony-forming unit fibroblast
HSCP	Hematopoietic stem cells and progenitors
HBO	Hyperbaric oxygen
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MSC	Mesenchymal stromal cell
OSX	Osterix
PPAR- γ	Peroxisome proliferator-activated receptor- γ
RUNX2	Runt-related transcription factor 2
SCP	Stem cells and progenitor
TNF- α	Tumor necrosis factor α

Background

Allogeneic bone marrow (BM) transplantation (BMT) results in more deaths, more tissue injury and higher pro-inflammatory response in old mice and humans than in the young (Ordemann et al. 2002). Advanced age is accompanied by a marked decrease in the number of CD34⁺ hematopoietic stem cells and progenitors (HSCPs) and attenuated lymphoid differentiation (Lansdorp et al. 1993; de Haan and Van Zant 1999; Lee et al. 2005). These phenomena suggest that self-renewal and proliferative potential of hematopoietic cells are diminished with age.

Among modalities studied for the potential to mitigate the problems associated with BMT is treatment by hyperbaric oxygen (HBO), a method effective in therapy that requires tissue regeneration (Neuman and Thom 2008). HBO influences tissues by different mechanisms, including modulation of the inflammatory response after BMT (Xiao-Yu et al. 2008) and mobilization of vasculogenic and HSCPs into circulation (Thom et al. 2006; Milovanova et al. 2009).

Although older subjects are more likely to require the benefit of HBO, the role of age on the effectiveness of HBO has not been explored. The need for understanding the role of aging is buttressed by the adverse events caused by the standard methods of HSCPs mobilization by chemotherapeutics and/or growth factors that increase the risk of acute arterial thrombosis, angina, sepsis, and death (Takahashi et al. 1999) in the elderly much more than in the young (Nomellini et al. 2009). The effects of age on the putative HBO-effects on mesenchymal stromal cells (MSCs) and

their differentiation potential are unknown. Consequently, we studied the effects of HBO on HSCPs and MSCs in a murine model.

Methods

Animals

Pathogen-free young (2 months) and old (18 months) female C57BL/6 mice from the National Institute of Aging colony at Harlan Laboratories (Indianapolis, IN) were maintained in an environmentally controlled facility at Mayo Clinic for at least one week prior to experiments. Immediately after death, mice were dissected and organs screened for visible tumors and/or gross abnormalities, but none was found. All experimental protocols followed the guidelines in “Principles of Laboratory Animal Care” (NIH publication No. 86-23, revised 1996) and were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Normobaric and HBO treatment

Mice (three to four animals per cage) were placed in an animal hyperbaric chamber (Mechidyne Systems, Inc. Houston, TX). Pressure of pure medical grade oxygen was increased to 2.8 atm absolute (ATA; 283.7 kPa) in the course of 6.5 min. After 90 min of HBO, pressure was reduced to 1.0 ATA (101.3 kPa) in 5.0 min (Quirinia and Viidik 1996; Thom et al. 2006). Control mice were exposed to air or normobaric oxygen to test the evidence that neither pure normobaric oxygen nor hyperbaric air had any effect on HSCPs numbers in circulation (Thom et al. 2006). The animals were exposed to five consecutive daily HBO treatments. Eighteen hours after the last treatment the mice were killed by CO₂ inhalation. The results shown are representative of three independent experiments.

Blood cell count

EDTA-anticoagulated blood was drawn from the right ventricle. Complete blood cell counts were quantified using a Hemavet 850FS cell counter (Drew Scientific, Oxford, CT).

Colony-forming cell assays

Individual hematopoietic colony-forming units in culture (CFU-C) were enumerated in peripheral blood and BM cells. A leukocyte suspension was prepared from EDTA-anticoagulated peripheral blood by lysing erythrocytes with ammonium chloride. BM was obtained by flushing femora with α -minimum essential medium containing 2% fetal bovine serum (FBS). One million leukocytes or 2×10^5 BM cells were plated in 35-mm dishes containing methylcellulose and growth factors (MethoCult GF M3434 assay; StemCell Technologies, Vancouver, Canada) according to manufacturer's protocol and placed at 37°C in humidified air containing 5% CO₂. At day 12, colonies were counted and evaluated using an inverted microscope.

MSC frequency was evaluated by colony-forming unit fibroblast (CFU-F) assay using the complete MesenCult mouse medium 05501 (StemCell Technologies) according to manufacturer's protocol. One million leukocytes or BM cells were plated in duplicate for each mouse and incubated in six-well plates (2.0 mL/well). Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C. After 10 to 13 days in culture, the medium was decanted, adherent colonies washed with PBS twice, air-dried for 5 min, covered with methanol and incubated at room temperature for 5 more minutes. Then methanol was decanted, colonies were air dried for 5 min and the well covered with Giemsa Staining Solution. After 5 min, the wells were washed with water and air-dried. Colonies were counted using an inverted microscope.

Isolation and culture of splenic macrophages

Splenic macrophages were isolated by plastic adherence (Boehmer et al. 2005; Gomez et al. 2010). Spleens were aseptically removed and the cells disassociated by passing through a nylon mesh in RPMI 1640 medium, supplemented with 5% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), and 2 mM glutamine (culture medium; GIBCO-BRL, Grand Island, NY). Following red blood cell lysis with ACK Lysis Buffer (Invitrogen, Carlsbad, CA), white blood cells were counted in a hemocytometer; their viability was determined by trypan blue exclusion. Two million cells/well were seeded in 96-well plates in 200 µL of culture medium. After incubation for two hours at standard tissue culture

conditions, non-adherent cells were aspirated and discarded; adherent cells were washed twice with warm phosphate buffer saline. This method resulted in adherent cells that were 98% positive for Mac-3 and Di-I-acetylated low-density lipoprotein uptake (Faunce et al. 1998). Adherent cells were treated in 200 µL of culture medium containing 100 ng/mL LPS from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO). Supernatants were collected after 18 h and stored at −80°C.

Measurement of pro-inflammatory cytokines

Concentrations of tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6) in macrophage supernatants were measured by commercial ELISA kits (OptEIA; BD Pharmingen, San Diego, CA) according to manufacturer's instructions. The lower detection limit of the kits was 15.6 pg/mL.

Real-time PCR for early differentiation markers

BM cells were cultured in complete MesenCult mouse medium 05501 for 10 days; the medium was replaced twice a week. Total RNA was isolated using TRIzol Reagent (Invitrogen) and quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). One microgram of RNA was reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen). Following reverse transcription, real-time PCR was performed using LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics, Indianapolis, IN). The forward and reverse primers were designed using the PrimerTime qPCR Assay (Integrated DNA Technologies, Skokie, IL). Primer sequences are shown in Table 1. Each primer master mix consisted of one forward and one reverse primer (10 µM each), SYBR Green I Master Mix (2× concentration), and sterile water. Five µL of the cDNA was aliquoted to each well of the LightCycler 480 Multiwell Plate (Roche Diagnostics); 15 µL of the primer master mix was added to cDNA. Each reaction was run in duplicate in a final volume of 20 µL. In the LightCycler 480 real-time PCR apparatus, the pre-incubation at 95°C took five min and was followed by 45 amplification cycles at 95°C for 30 s and then 60°C for 30 s. The results for individual genes were normalized based on the expression of the housekeeping gene TATA-binding protein (Syed et al. 2010) run in the same plate. Data are expressed as the

Table 1 Primer sequences employed in analysis of transcript levels for early markers of adipocytic and osteoblastic differentiation of mesenchymal stromal cells

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
PPAR- γ	GAGGAGTCCCTTCCCTCATC	TCCTCGAAGGTTAAGGCTGA
C/EBP- β	GTTTCGGGACTTGATGCAAT	GCCCGGCTAGACAGTTACAC
aP2	GAATGTGTTATGAAAGGCGTGAC	AAATTTCCATCCATCCAGGCCTCT
RUNX2	CCAGGAAGACTGCAAGAAGG	TCCTGCATGGACTGTGGTTA
OSX	GGAGGTTTCACTCCATTCCA	TAGAAGGAGCAGGGGACAGA
AP	CACAGATTCCCAAAGCACCT	GGGATGGAGGAGAGAAGGTC
TBP	CTCAGTTACAGGTGGCAGCA	CAGCACAGAGCAAGCAACTC

Markers of adipocytic differentiation: *PPAR- γ* Peroxisome proliferator-activated receptor- γ , *C/EBP- β* CCAAT/enhancer binding protein- β , *aP2* fatty-acid binding protein 4. Markers of osteoblastic differentiation: *RUNX2* runt-related transcription factor 2, *OSX* osterix, *AP* alkaline phosphatase. Housekeeping gene: *TBP* TATA-binding protein

factor (“fold”) of change relative to the expression in young control animals.

Statistical analysis

Groups consisted of no more than four mice. The total number of animals studied was 13, 3 and 7 young mice in normobaric air, normobaric oxygen, and HBO, respectively and 9, 3 and 10 old mice in normobaric air, normobaric oxygen, and HBO, respectively. As the number of animals in study groups differed, we analyzed the data using a two-way ANOVA of logarithmically transformed data. *p* values were obtained as pair-wise comparisons between group means using the Fisher's Least Significant Difference method. Because the overall global *p* value was 0.009, the Fisher's Protected Least Significant Difference method provided correct control over type I error (false positives); hence, the pair-wise *p* values less than 0.05 could be considered statistically significant.

Results

HBO affects circulating blood cells in young, but not old mice

To determine the effects of age and HBO on circulating cells, we exposed mice to pure oxygen at 2.8 ATA for 90 min on each of five consecutive days and compared the effects to control mice breathing normobaric air (Table 2). HBO-treated young mice exhibited an increase in lymphocyte and monocyte counts relative to control air-breathing mice

(*p* < 0.05), but blood counts of total white blood cells, basophils, eosinophils, red blood cells and platelets did not differ. Peripheral blood counts in old air-breathing mice were similar to young air-breathing mice, except for elevated monocyte and platelet levels (*p* < 0.05). However, HBO did not affect the cell densities in old mice.

Effects of age on hematopoietic progenitors

Because HBO, but not normobaric oxygen, mobilizes SCPs in young mice (Thom et al. 2006; Milovanova et al. 2009), we explored the effects of HBO on SCP mobilization in old mice by determining the number of hematopoietic colony forming units in culture (CFU-C). For additional control, we exposed the animals to normobaric oxygen under conditions otherwise identical to those exposed to HBO. We found no effect of normobaric oxygen compared to normobaric air in the young and old (*p* > 0.05), but the blood of old control mice yielded twice as many CFU-Cs as the blood of young mice (*p* < 0.05; Fig. 1). HBO treatment doubled this number in young mice (*p* < 0.05); the increase in old mice did not reach statistical significance. The number of CFU-Cs from BM cells was higher in old control mice than in young control mice (*p* < 0.05; Fig. 1). HBO effect on BM cells was similar to the effect on circulating cells: it affected young mice (*p* < 0.05), but not old mice. Again, normobaric oxygen had no effect in either group (*p* > 0.05). These results suggest that aging increases the number of CFU-C-generating cells on its own and that HBO has no effect on these cells in old mice.

Table 2 Effects of age on circulating blood cells in young and old mice

	Young		Old	
	Air	HBO	Air	HBO
White blood cells	5.5 ± 0.6	8.5 ± 0.6	6.2 ± 1.1	6.1 ± 0.8
Lymphocytes	4.2 ± 0.5	6.5 ± 0.4*	3.8 ± 0.7	4.0 ± 0.4
Monocytes	0.2 ± 0.0	0.3 ± 0.0*	0.3 ± 0.0**	0.3 ± 0.0
Neutrophils	1.2 ± 0.1	1.7 ± 0.2	1.9 ± 0.4	1.3 ± 0.1
Basophils	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Eosinophils	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Red blood cells	7.8 ± 0.1	8.0 ± 0.4	7.9 ± 0.1	7.3 ± 0.3
Platelets	648 ± 26.8	722 ± 40.9	954 ± 24.1**	900 ± 74.5

Young and old mice were treated by 100% oxygen at 2.8 ATA for 90 min on five consecutive days. Controls breathed normobaric air. Complete blood counts were determined using a Hemavet 850FS cytometer (DREW Scientific, Oxford, CT). All cell numbers are expressed as thousands per μL and shown as mean \pm SEM. * Significant compared to animals breathing normobaric air. ** Significant compared to young animals breathing normobaric air. For lymphocytes, $p < 0.05$ for the difference between young controls and young hyperbaric oxygen (HBO)-treated animals; for monocytes, probabilities for comparisons of control and HBO-treated young mice and young control mice versus old control were < 0.05 as was the probability for the difference of platelet numbers between young controls and old controls

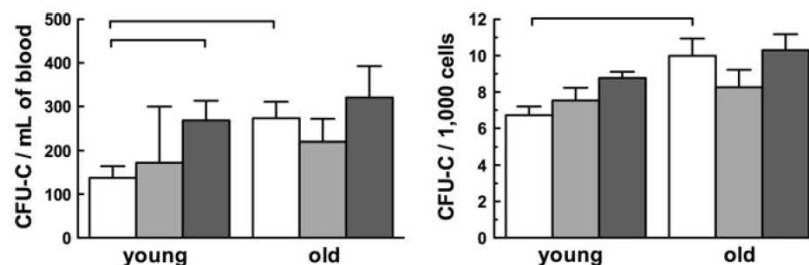


Fig. 1 Hyperbaric oxygen and age affect hematopoietic propensity of circulating and bone marrow cells. Mice breathed normobaric air (controls; *white bars*) or normobaric oxygen (lightly shaded bars) or hyperbaric oxygen (HBO; *heavily shaded bars*) for five consecutive days. Eighteen hours after final HBO treatment, animals were killed and hematopoietic stem cells and progenitors (HSCPs) were quantified as colony-forming units in culture (CFU-C) by peripheral blood leukocytes (*left panel*) and bone marrow (BM) cells (*right panel*).

Mice were treated in groups of no more than four. The total number of animals studied was 13, 3 and 7 young mice in normobaric air, normobaric oxygen, and HBO, respectively and 9, 3 and 10 old mice in normobaric air, normobaric oxygen, and HBO, respectively. Pooled data for all mice that underwent the same treatment are shown as mean values \pm SEM. *Horizontal lines* indicate the differences among groups that are statistically significant ($p < 0.05$)

As normobaric oxygen did not affect proliferation as the ultimate measure of cell function, we focused further studies on the effects of HBO.

HBO suppresses expression of proinflammatory cytokines by activated macrophages

Most CFU-Cs developed from blood and BM cells were CFU-granulocyte/macrophage (data not shown); this indicated that most cells were committed to differentiation into myeloid cells. This observation

compelled us to determine the effects of HBO on myeloid cell function. We isolated splenic macrophages, activated them by lipopolysaccharide (LPS) and measured expression of IL-6 and TNF- α , cytokines previously tested in studies of HBO effects (Lahat et al. 1995; van den Blink et al. 2002; Benson et al. 2003; Fildissis et al. 2004; Buras et al. 2006; Thom 2009). We found no detectable cytokines in the media conditioned by LPS-free macrophages (data not shown), but in the presence of LPS macrophages expressed high levels of IL-6 and TNF- α (Fig. 2). In

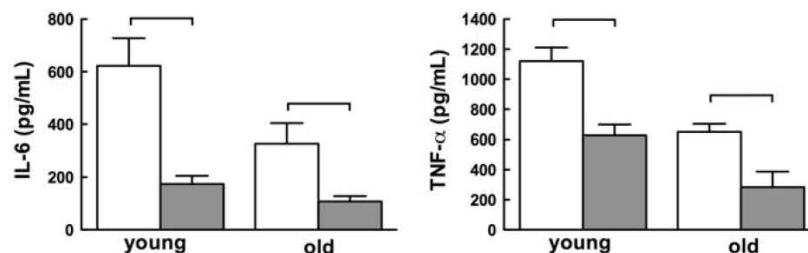


Fig. 2 Hyperbaric oxygen suppresses secretion of inflammatory cytokines. Splenic macrophages obtained from young and old mice shown in Fig. 1 were cultured for 18 h with lipopolysaccharide (LPS; 100 ng/mL). Supernatants were assayed for interleukin-6 (IL-6) (*left panel*) and TNF- α (*right*

panel). Data are shown as mean values \pm SEM for all mice that underwent the same treatment. *Horizontal lines* indicate the differences among groups that are statistically significant ($p < 0.05$)

agreement with others, macrophages from old mice secreted less of these cytokines (Renshaw et al. 2002; Boehmer et al. 2005; Chelvarajan et al. 2005; Gomez et al. 2010). HBO reduced the mean concentration of IL-6 by 72% in the media conditioned by macrophages from young mice ($p < 0.05$) and by 67% by macrophages from old mice ($p < 0.05$). TNF- α secretion was similarly reduced by 44% ($p < 0.05$) and 56% ($p < 0.05$), respectively. These results show that systemic HBO exposure reduces in vitro pro-inflammatory cytokine expression in macrophages from young and old mice alike.

Effect of age on HBO on MSCs

Based on the recent interest in the potential role of MSCs in regenerative medicine, we set to determine if HBO affects this major group of cells as well. Consequently, we quantified the MSC frequency in peripheral leukocytes and BM by the CFU-F assay. We found no CFU-F-generating cells in the blood of young air-breathing and HBO-treated mice. However, leukocytes of old air-breathing mice yielded low, but detectable numbers of CFU-Fs (1.0 ± 0.8 CFU-F/ 1×10^6 leukocytes); the number of CFU-Fs did not change by HBO ($p > 0.05$).

In distinction to leukocytes, BM cells gave rise to typical CFU-Fs (Fig. 3). One million BM cells of young mice yielded 4.5 ± 0.8 CFU-Fs (Fig. 4). Surprisingly, HBO doubled the number of CFU-Fs in young mice ($p < 0.05$). Less unexpected was the finding that the number of CFU-F-generating cells in the BM of old mice was fourfold higher relative to young animals ($p < 0.051$), while HBO did not affect

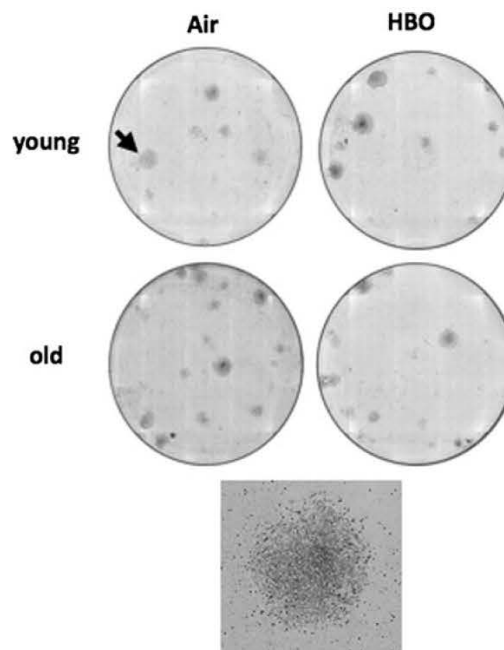


Fig. 3 Age and HBO affect bone marrow-derived MSCs. MSC frequency in leukocytes and BM was evaluated by colony formation. One million leukocytes or BM cells from animals breathing normobaric air or treated by HBO were cultured for up to 13 days and stained by Giemsa when CFU-fibroblast (CFU-F) colonies were counted under the microscope. Shown are representative Giemsa-stained plates of BM-derived CFU-Fs; the arrow in the upper left plate points to the randomly selected colony that is magnified in the square panel to demonstrate the morphology typical for CFU-Fs

that number ($p > 0.05$). These findings suggest that HBO affects BM MSC and that the number of CFU-F-generating cells increases with age.

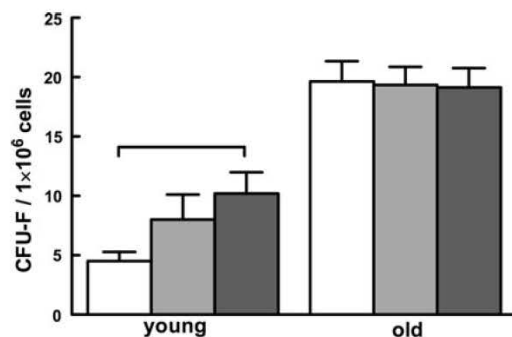


Fig. 4 Hyperbaric oxygen and age affect the frequency of mesenchymal stromal cells in bone marrow. Mesenchymal stromal cells (MSCs) from mice in Fig. 1 were quantified as CFU-fibroblast (CFU-F) colonies. Horizontal lines indicate the difference between groups that is statistically significant ($p < 0.05$)

HBO modifies expression of early differentiation genes in MSCs

To gain insight whether age-related and HBO-related changes in CFU-F-generating cells in Figs. 3 and 4 are

reflected in the differentiation potential of these cells, we analyzed transcript levels of genes associated with early differentiation into adipocytes and osteoblasts. Genes of adipocytic differentiation (Gregoire et al. 1998) included the peroxisome proliferator-activated receptor- γ (PPAR- γ); CCAAT/enhancer binding protein- β (C/EBP- β); and fatty-acid binding protein 4 (aP2); while those of osteogenic differentiation (van Straalen et al. 1991; Karsenty 2008) were the runt-related transcription factor 2 (RUNX2); osterix (OSX); and alkaline phosphatase (AP). We measured transcript levels by quantitative PCR and for each transcript normalized the data of all groups to its expression level in young air-breathing mice. Interestingly, expression of PPAR- γ and C/EBP- β was affected neither by age nor by HBO, while the level of aP2 transcripts in old mice was elevated relative to young mice ($p < 0.05$); the reduction by HBO did not reach statistical significance (Fig. 5). In contrast, osteoblastic differentiation markers were affected both by age and HBO. The levels of gene transcripts in old mice were threefold, 19-fold and fourfold higher for RUNX2, OSX and AP, respectively ($p < 0.05$ for all three transcripts).

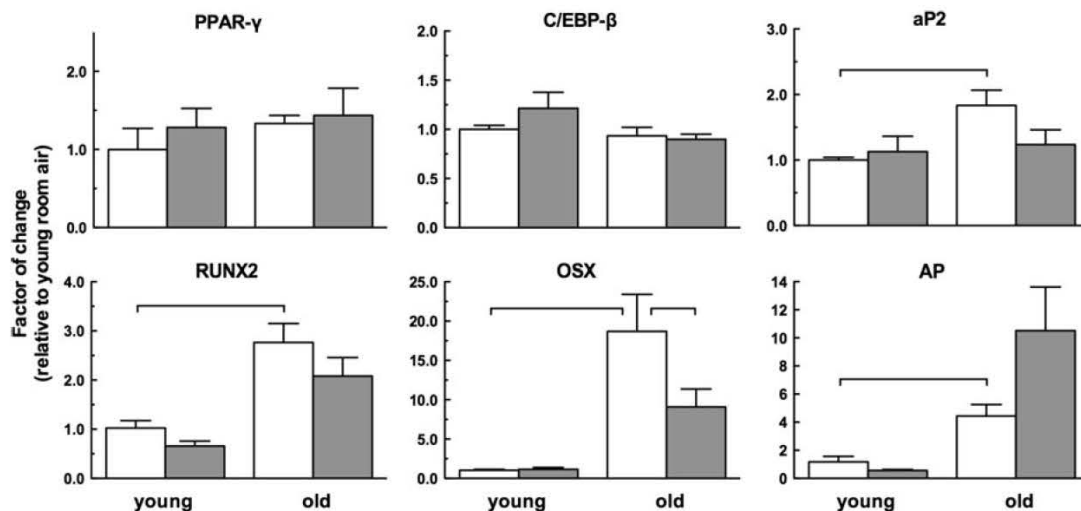


Fig. 5 Hyperbaric oxygen modulates transcription of osteogenic, but not adipogenic differentiation genes in bone marrow mesenchymal stromal cells of old mice. Transcripts associated with early adipogenic differentiation [peroxisome proliferator-activated receptor- γ (PPAR- γ); CCAAT/enhancer binding protein (C/EBP- β); and, fatty acid binding protein 4 (aP2)];

and early osteogenic differentiation [runt-related transcription factor 2 (RUNX2); osterix (OSX); and, alkaline phosphatase (AP)] were quantified by real-time PCR in bone marrow mesenchymal stromal cells obtained from mice in Fig. 1. Horizontal lines indicate the differences among groups that are statistically significant ($p < 0.05$)

While the effects of HBO on transcript levels were marginal in young mice, they were prominent in the old. There was no change in *RUNX2* levels, levels of transcripts for *OSX* were reduced by one half ($p < 0.05$) in contrast to doubling of *AP* transcripts (not statistically significant). Overall, these results indicate age and HBO affect the expression of osteoblastic early differentiation genes in MSCs.

Discussion

The purpose of this work is to probe the effects of senescence on the ability of HSCPs and MSCs to respond to HBO. The impetus for the study is the need to understand if efficacy of the standard HBO therapy protocols depends on age and, hence, the protocols might have to be tailored to patient age. Based on the general understanding of differences in HSCPs and MSCs between the young and the old (Lansdorp et al. 1993; de Haan and Van Zant 1999; Lee et al. 2005; Sethe et al. 2006; Gazit et al. 2008; Roobrouck et al. 2008), the underlying hypothesis has been that HBO may affect HSCPs and MSCs in an age-dependent manner.

For this study we selected 2-month old and 18-month old mice. Based on the allometric relationships between body size and expected longevity, the younger group would be roughly equivalent to human adolescents, while the older to an octogenarian (Lindstedt and Calder 1981). In this study we focused on a single gender of mice (females); additional experiments in males should define the effect of age and gender on HBO-mediated mobilization of SCPs. Some gender-specific differences might be anticipated based on gender-related response of old mice to stressors (Gomez et al. 2009).

Using the selected model of senescence, we confirmed that the levels of CFU-C-forming cells in the blood and marrow of old mice were higher than in the young mice. A pertinent finding is that HBO affected the proliferative capacity of HSCPs and MSCs in the young, but not in old mice, in line with the observation that systemic hyperoxia enhances proliferation of human (Thom et al. 2006) and murine BM-derived HSCPs (Thom et al. 2006; Milovanova et al. 2009). After a single HBO treatment Thom et al. detected a twofold increase in CFU-Cs in murine peripheral blood leukocytes and BM cells. They found HSCP

mobilization by measuring expression of CD34 and Sca-1 in peripheral blood cells (Thom et al. 2006; Milovanova et al. 2009); our results confirm the latter finding by colony forming and demonstrate that the cells are functional and add considerable weight to evidence that HBO mobilizes HSCPs.

Mobilizing agents induce rapid emigration of stem cells from the BM. However, under most circumstances mobilization requires cell proliferation in the BM (Nakamura et al. 2004). The higher basal level of HSCPs in old mice could be associated with the age-related expansion of the SCP pool that originates in the increased autonomous cell renewal capacity (Gazit et al. 2008). In contrast, old age is linked to a decline in SCP function (Sudo et al. 2000; Geiger and Van Zant 2002), including less efficient hematopoiesis (Chen et al. 2000), reduced differentiation potential (Rossi et al. 2005) and impaired homing (Xing et al. 2006). Altogether, these studies suggest that age-related reduction of HSCP activity could be offset by the increase in HSCP numbers (Gazit et al. 2008). Our results are in line with other phenomena in aging, e.g., the pro-inflammatory phenotype, even in the absence of insult (“inflamm-aging”) that results in an inherent activation rendering the system less able to respond to perturbation (de Haan and Van Zant 1999; Franceschi et al. 2000; Gomez et al. 2005).

Our finding that HBO does not mobilize HSCPs in the old is at variance with mobilization by granulocyte-colony stimulating factor (Xing et al. 2006) that reduces HSCP adhesion to BM stroma even in the old (Geiger and Van Zant 2009). This difference suggests qualitatively and/or quantitatively distinct mechanisms in HBO-mediated and G-CSF-mediated HSCP mobilization in old mice. In response to HBO, nitric oxide levels increase and trigger a cascade mechanism that mobilizes SPCs from BM by the release into circulation of cytokines such as cKit ligand (stem cell factor, SCF; Thom et al. 2006). The effects of aging on this mechanism are unknown, but HSPC mobilization by chemotherapeutics and/or growth factors increases the risk of acute arterial thrombosis, angina, sepsis, and death (Takahashi et al. 1999) in the old much more than in the young (Nomellini et al. 2009). Consequently, it will be important to clarify the effects of aging on the components of the proposed HBO-mediated cascade involved in SCP mobilization. Additional insight might be gained from studies of the effects of aging and HBO on the expression of

SDF-1 and CXCR4, the critical regulators of SPC function and homing, and of nitric oxide-mediated mechanism of HSCP mobilization by HBO.

Aging increases the differentiation potential of myeloid cells [reviewed in (Linton and Dorshkind 2004)], but results also in their functional defects [reviewed in (Gomez et al. 2008)]. Our studies confirmed that LPS-activated macrophages from old mice express less proinflammatory cytokines IL-6 and TNF- α relative to young cells (Renshaw et al. 2002; Boehmer et al. 2005; Chelvarajan et al. 2005; Gomez et al. 2010). Interestingly, HBO reduced IL-6 and TNF- α expression to a similar extent in the young and the old, in line with some (Benson et al. 2003; Buras et al. 2006; Thom 2009), but not all observations (Lahat et al. 1995; van den Blink et al. 2002; Fildissis et al. 2004). Apparently, HBO can control acute inflammation following injury and sepsis (Huang et al. 2005; Oter et al. 2005; Neuman and Thom 2008), but its effects on increased morbidity and mortality associated with aberrant inflammatory responses in the old are largely unknown and, therefore, very much worthy of additional research. While HBO-mediated mobilization of HSCPs into circulation has been known for some time (Thom et al. 2006), HBO effects on MSCs have not been studied. We found no CFU-F-forming cells among peripheral blood leukocytes under any condition, except in old mice where we did detect low levels of these cells. The significance of this observation will require further study. However, interestingly in BM, HBO increased the CFU-F number in young mice.

MSCs and the effects of aging on them (Sethe et al. 2006; Roobrouck et al. 2008) are gaining interest because of the putative applicability of MSCs in regenerative therapy and tissue engineering (Parekkadan and Milwid 2010). We found detectable levels of circulating MSCs (measured by the ability to generate CFU-F colonies) only in old air-breathing mice. In the BM, these cells were detectable in young mice and at a fourfold higher level in old mice. This result differs from the reports of age-related decrease in the total number of CFUs or the absence of age effects [reviewed in (Sethe et al. 2006)]. Further MSC evaluation by techniques like flow cytometry may be needed to detect fractions of MSCs especially sensitive to the culture conditions used in CFU-F assays and to settle the discord among the results.

The effects of oxygen tension on MSCs have not been clearly established either. On one side, hypoxia decreased proliferation and differentiation of BM-derived MSCs (Mohyeldin et al. 2010). On the other side, HBO-stimulated bone formation and healing (processes involving MSCs) did not enhance the osteogenic ability of MSCs in spinal fusion in a rabbit model (Fu et al. 2010). We found that HBO increased the number of CFU-Fs in the young BM. We demonstrated that young MSCs retained their differentiation potential (measuring adipogenic differentiation; data not shown), but that HBO had no effect on this process. This observation adds to those who suggest that in young mice HBO can change the numbers of BM-derived MSCs without affecting their differentiation potential (Fu et al. 2010).

Lineage commitment of MSCs is controlled by an array of intracellular and extracellular signals in the BM milieu, including the activation of phenotype-specific transcription factors (Karsenty 2008). Our results show that transcript levels of genes involved in adipogenic differentiation were unaffected by age, but that transcript levels of early osteoblastic differentiation genes were higher in old MSCs. These results differ from description of higher levels of aP2 transcripts (an adipocyte differentiation gene) in MSCs from old mice (no gender specified) and lower levels of transcripts of osteoclast-specific transcription factors including RUNX2 (Moerman et al. 2004). However, our findings are in line with the expression of significantly higher transcript levels for all osteoblast differentiation marker genes in hematopoietic lineage-negative (lin^-) cells from old female C57/BL6 mice (Syed et al. 2010). Since lin^- cells are highly enriched in osteoblastic progenitors that can mineralize in vitro, form bone in vivo, and express bone-related genes, our results are comparable with the demonstration of aging effects on osteoblast progenitors (Syed et al. 2010). Quantifying the baseline expression of osteogenic and adipogenic differentiation markers provided a snapshot of the effects of aging and HBO on the MSC differentiation potential. Expression of these genes during MSC differentiation coupled with other differentiation assays (e.g., in vitro mineralization and in vivo bone formation for osteoblasts and in vitro lipid formation for adipocytes) will provide more definite evidence for the effects of age and HBO on lineage commitment by MSCs.

Observation of MSC-restricted expression of early differentiation genes affected by HBO in old mice only warrants in-depth studies of the involved intracellular signaling networks. Study of signaling involving extracellular mediators, in particular the transforming growth factor- β , bone morphogenetic proteins 2 and 4 with a known role in controlling adipocyte versus osteoblast formation (Gregoire et al. 1998; Karsenty 2008) will help define the mechanisms involved in the effects of HBO in MSCs, particularly in the old.

In a murine model of aging we studied the effects of HBO on HSCPs and MSCs. We demonstrated that aging affected the ability of mice to mobilize HSCPs from the BM and suggest an age-related defect in mobilization by HBO; mechanistic aspects of this phenomenon require further elucidation. Our results suggest that HBO therapy protocols may have to be adjusted by age or eventually individualized. In addition, these results indicate the potentially different benefit of HBO in wound healing and tissue remodeling in the old and the young.

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Original article

Dysregulation of Neutrophil CXCR2 and Pulmonary Endothelial ICAM-1 Promotes Age-Related Pulmonary Inflammation

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ABSTRACT: We have previously demonstrated that aging is associated with prolonged pulmonary inflammation in a murine model of thermal injury. To further investigate these observations, we examined lung congestion, markers of neutrophil chemotaxis and adhesion, and lung endothelial responses in young and aged mice following burn injury. Analysis of lung tissue and bronchoalveolar lavage fluid 24 hours after injury revealed that more neutrophils accumulated in the lungs of aged mice ($p < 0.05$), but did not migrate into the alveoli. We then sought to determine if accumulation of neutrophils in the lungs of aged mice was due to differences in the peripheral neutrophil pool or local changes within the lung. Following burn injury, aged mice developed a pronounced peripheral blood neutrophilia ($p < 0.05$) in comparison to their younger counterparts. In aged animals, there was a reduced frequency and mean fluorescent intensity of neutrophil CXCR2 expression ($p < 0.05$). Interestingly, in uninjured aged mice, peripheral blood neutrophils demonstrated elevated chemokinesis, or hyperchemokinesis, ($p < 0.05$), but showed a minimal chemotactic response to KC. To determine if age impacts neutrophil adhesion molecules, we assessed CD62L and CD11b expression on peripheral blood neutrophils. No age-dependent difference in the frequency or mean fluorescent intensity of CD62L or CD11b was observed post-burn trauma. Examination of pulmonary vasculature adhesion molecules which interact with neutrophil selectins and integrins revealed that intracellular adhesion molecule-1 (ICAM-1) was elevated in aged mice at 24 hours after burn as compared to young mice ($p < 0.05$). Overall, our data suggests that age-associated pulmonary congestion observed following burn injury may be due to differences in lung endothelial adhesion responses that are compounded by elevated numbers of hyperchemokinetic circulating neutrophils in aged mice.

Key words: Adhesion; Aging; Chemotaxis; Neutrophils; Lung; Burn injury

While the mortality of burn patients over the age of 65 has improved over the last few decades, clinical outcomes are still very poor [1, 2]. As the proportion of elderly individuals continues to rise, this issue will translate into a greater socioeconomic burden. It is therefore important that new treatment strategies are

developed to minimize the effects of age on the response to burn and other forms of traumatic injury.

Similar to other insults which lead to systemic inflammation, the development of pulmonary complications, such as pneumonia and acute respiratory distress syndrome, are often the most serious threat to

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the burn patient and are especially detrimental to the elderly [3-5]. In contrast to most other organs in the body, the lung has two potential routes for an inflammatory insult: through the airway and through the bloodstream. Interestingly, the pathogenesis of pulmonary inflammation is dependent on where the stimulus is located. When an inflammatory source is located in the airway, a rapid neutrophil recruitment to the alveolar space is observed [6-12]. However, when the source originates elsewhere in the body and disseminates through the blood, neutrophil accumulation within the pulmonary vasculature occurs, but cells do not migrate into the alveoli; here, the neutrophils are said to be "sequestered" in alveolar capillaries [13-15]. The difference between these two host responses are established by multiple factors including chemokine gradients and expression of adhesion molecules [7, 16, 17].

Regardless of the mechanism of pulmonary inflammation, neutrophil chemokines are important in neutrophil recruitment in response to local or systemic injury. In mice, the main chemokines involved in the process are macrophage inflammatory protein-2 (MIP-2, or CXCL2) and KC (or CXCL1), analogues of human growth-related oncogene α and β , respectively [18, 19]. These chemokines both bind to the receptor, CXCR2, on neutrophils [20, 21]. Not only do these chemokines act to stimulate neutrophil chemotaxis towards an inflammatory stimulus, but also to induce firm adhesion to the endothelium and to mediate diapedesis [19, 21, 22]. These studies also imply that there is a sequential order of signaling required for neutrophil diapedesis involving both selectins (CD62L) and integrins (CD11/CD18) [19, 23]. Following ligation of the neutrophil chemotactic receptor CXCR2, activation of the small GTPase: Ras-related protein-1 (Rap1) and phospholipase C (PLC) promotes upregulation of neutrophil selectins and integrins [24-26]. Subsequent interaction and cross-linking of CD62L, further upregulates integrin activity and allows neutrophils to roll and loosely adhere to the vascular endothelium [27, 28]. Together, this results in clustering of CD11/CD18, as well as other adhesion molecules, and promotes firm adhesion of the neutrophils to the endothelium via intracellular adhesion molecule-1 (ICAM-1) [17]. Neutrophils can then interact with chemokines immobilized on the apical side of endothelial cells and begin the process of diapedesis [21, 29, 30].

Previously, our laboratory was the first to show that aged mice have prolonged pulmonary inflammation after burn injury compared to young mice with a comparable insult, marked by neutrophil accumulation and increased KC levels [31]. Herein we demonstrate that following burn trauma, aged mice have an elevated number of

neutrophils in both the peripheral blood and lung that is unrelated to differences in neutrophil chemotactic or adhesion markers. Moreover, our data suggest that an age-associated elevation in lung endothelial ICAM-1 may promote the observed pulmonary congestion.

MATERIALS AND METHODS

Animals

Young (2-6 months) and aged (18-22 months) female BALB/c mice were obtained from the National Institute of Aging colony at Harlan Laboratories (Indianapolis, IN) and maintained on a 12 hour light/dark cycle with standard laboratory rodent chow and water ad libitum. All experimental procedures were performed according to the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, and approved by the Animal Care and Use Committee at Loyola University Medical Center.

Induction of Burn Injury

As previously described, mice were anesthetized with pentobarbital (50 mg/kg i.p.), shaved and placed into a plastic template designed to give a 15% total body surface area, full-thickness dorsal scald injury when immersed in a boiling water bath for 8 seconds [31, 72, 73]. As a control, separate groups of young and aged mice received a sham injury, in which they were administered anesthesia and shaved, but immersed in a room temperature water bath. Immediately following injury, the mice received warm saline resuscitation and their cages were placed on heating pads until fully recovered from anesthesia. Mice were sacrificed using CO₂ inhalation and cervical dislocation. In addition, all mice—including those which died before the time of sacrifice—were examined for visible tumors and, if found, were removed from the study.

Bronchoalveolar Lavage

To determine the cell populations in the alveolar space, bronchoalveolar lavage (BAL) was performed on young and aged mice 24 hours after receiving either a burn or a sham injury [6][2]. Immediately following sacrifice, the trachea was exposed and a small incision was made just below the cricothyroid cartilage. The trachea was then cannulated using 22 gauge needles and 1 ml of phosphate buffered saline was repeatedly injected until 5 ml of fluid was recovered for each animal. BAL cells were immunostained for flow cytometry analysis, as described below.

Isolation of Peripheral Blood Neutrophils

Blood was taken via cardiac puncture of the left ventricle. For chemotaxis assays, samples were diluted

1:1 in Hank's Buffered Saline Solution (HBSS), layered on Histopaque 1083 (Sigma, St. Louis, MO), and centrifuged at 400 g for 30 minutes at 20°C without the brake applied. The monocyte and plasma layers were aspirated, leaving granulocytes and erythrocytes. Samples were resuspended in HBSS and 3% dextran was added to sediment the erythrocytes. After 45 minutes at room temperature, the top layer containing granulocytes was removed and centrifuged at 300 g for 5 minutes. Any remaining erythrocytes were lysed using ACK buffer (Invitrogen, Carlsbad, CA).

Chemotaxis Assay

Neutrophils were isolated as described above. Chemotaxis assays were then performed as described by others [52, 74]. Neutrophils were centrifuged and resuspended in 40 μ M of Cell Tracker Green (Invitrogen) in chemotaxis media containing HBSS, 25 mM HEPES and 1% BSA at 10^6 cells/ml. The cells were then incubated in the dark for 45 minutes at 37°C and 5% CO₂. After washing, the cells were resuspended in chemotaxis media at 10^6 cells/ml. The bottom wells of a chemotaxis chamber (NeuroProbe, Gaithersburg, MD) were filled with various concentrations of recombinant mouse KC (R&D Systems). A separate set of wells were filled with media alone as a negative control or 10^{-7} M fMLP (Sigma) as a positive control. Another set of wells were filled with sample inputs to determine the fluorescence of the starting cell suspension. A filter membrane containing 8 μ m pores (NeuroProbe) was then placed over the wells and cell suspensions were added to the upper side of the membrane at 10^6 /ml. Samples were incubated for 60 minutes at 37°C and 5% CO₂. Cell suspensions were then aspirated off the top membrane and 20 μ M EDTA was added to the upper side of the membrane for 15 minutes to allow any cells adhering to the membrane to detach. The membrane was then removed and the fluorescence of the bottom wells was measured in a fluorescence spectrophotometer. The percent of cells migrating was determined by comparing the fluorescence of the cells in the sample wells to that of the input wells.

Flow Cytometry

Analyses utilizing flow cytometry were performed as previously described [75, 76]. Cells were washed with HBSS and blocked with anti-CD16/32 antibody for 30 minutes at 4°C. Cells were then stained for 30 min at 4°C using anti-mouse antibodies at saturating concentrations, washed twice and fixed with 1% paraformaldehyde. Fluorescence was measured by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA). Anti-mouse antibodies were used at the following

concentrations: 2 μ g/ml of PE-conjugated rat anti-mouse Gr-1 (Invitrogen), 20 μ g/ml of APC-conjugated rat anti-mouse F4/80 (eBioscience, San Diego, CA) 10 μ g/ml of FITC-conjugated rat-anti mouse Gr-1 (eBioscience), 12.5 μ g/ml of PE-conjugated rat anti-mouse CXCR2 (R&D Systems, Minneapolis, MN), and 10 μ g/ml of PE-conjugated rat anti-mouse CD11b (eBioscience). Flow cytometry data were analyzed using FlowJo (Tree Star, Inc., Ashland, OR).

Immunofluorescence

The lungs were removed at the time of sacrifice, inflated with 25% O.C.T. freezing medium, and embedded for frozen sectioning as previously described [31]. Tissue sections were fixed in acetone and blocked with normal goat serum. To determine neutrophil content in lungs, sections were first incubated with 1 μ g/mL of rat anti-Gr-1 antibody (Invitrogen, Carlsbad, CA) followed by 4 μ g/ml of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen). Since Gr-1 can also be found on certain macrophage populations [3, 4], the sections were dual-stained with 0.2 μ g/mL of biotinylated anti-MOMA-2 antibody (BMA Biomedicals, Augst, Switzerland), a pan-macrophage marker, and detected with 2 μ g/ml of Cy3 Streptavidin (Invitrogen). Using fluorescent microscopy, the total number of neutrophils (designated as Gr-1⁺ MOMA-2⁻ cells) were counted across 10 high power fields for each animal [5]. Data are expressed as mean number of neutrophils counted in ten 400x fields \pm SEM. The total tissue area across which cells were counted was quantified and determined to be consistent between animals in all treatment groups (data not shown).

To determine the expression of endothelial ICAM-1 in the lungs after burn or sham injury, sections were incubated with 0.25 μ g/ml of Armenian hamster anti-mouse ICAM-1 (BD Pharmingen, San Diego, CA), followed by 3 μ g/ml of goat anti-Armenian hamster IgG conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA). Since ICAM-1 is also expressed on lung epithelium, sections were dual stained with 0.16 μ g/ml of rat anti-mouse platelet-endothelial cell adhesion molecule-1 (PECAM-1, BD Biosciences), and subsequently by 4 μ g/ml of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen). Expression of ICAM-1 on lung endothelium was determined by quantifying the total area of ICAM-1 and PECAM-1 colocalization across ten 400x fields per animal. Colocalization is expressed as the area of ICAM-1⁺PECAM-1⁺ (μ m²) staining. All fluorescent images were acquired using a Zeiss Axiovert 200 microscope (Zeiss, Germany).

Table 1. Neutrophil localization in lungs at 24 hours after burn

Method	Young		Aged	
	Sham	Burn	Sham	Burn
Total # Gr-1 ⁺ MOMA-2 ⁺ cells in 10 fields by immunofluorescence	16.0 ± 2.4	16.1 ± 2.4	12.8 ± 2.5	63.3 ± 11.5 *
% Gr-1 ⁺ F4/80 ⁺ cells ^b in lung homogenates by flow cytometry	2.0 ± 0.4	1.8 ± 0.3	2.1 ± 0.2	11.8 ± 3.0 ‡
% Gr-1 ⁺ F4/80 ⁺ cells ^c in BAL by flow cytometry	4.1 ± 3.5	0.4 ± 0.1	7.0 ± 2.7	0.5 ± 0.2 §

^a Total numbers of Gr-1⁺ MOMA-2⁺ cells in lungs of young and aged animals at 24 hours after sham or burn injury were counted in sections of lung tissue. Data are shown as the average number of cells counted in ten 400x fields for each group ± SEM. N = 8-14 mice per group; *p < 0.05 compared to all other groups.

^b All five lung lobes were homogenized in HBSS as described above. Cells were stained for Gr-1 and F4/80 as described and analyzed by flow cytometry. Data are shown as the average percent of Gr-1⁺ F4/80⁺ cells in the whole cell suspension ± SEM. N = 6-8 mice per group; ‡p < 0.05 compared to all other groups.

^c Lungs were lavaged with 1 ml of saline until 5 ml of sample was collected. Cells were spun down and stained with Gr-1 and F4/80 as described and analyzed by flow cytometry. Data are shown as the average percent of Gr-1⁺ F4/80⁺ cells in BAL ± SEM, §p < 0.05 compared to age-matched control.

Statistical Analysis

Data were analyzed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) and are expressed as mean ± SEM. A one-way ANOVA with Tukey's post-hoc was used to determine statistical differences between all groups. For comparisons of two groups, an unpaired Student's t-test was used. Statistical significance was set at p < 0.05.

RESULTS

Localization of neutrophils in the lungs of aged mice after burn

We previously reported that neutrophils are cleared from the lungs of young mice at 24 hours after burn while these cells are persistently elevated in the lungs of aged mice receiving the same injury [31]. Multiple methods were employed to determine the pulmonary compartment in which the neutrophils are located in the lungs of young versus aged mice at 24 hours after burn. Lung sections were co-immunostained with anti-Gr-1 and anti-MOMA-2 antibodies, and Gr-1⁺MOMA-2⁺ cells were considered neutrophils [32, 33]. Quantification of the number of neutrophils in lung sections from each group is shown in Table 1. At 24 hours after burn injury, there was a 4-fold increase in neutrophil numbers in aged mice subjected to burn injury compared to sham and young burn groups (p < 0.05). These data further corroborate our previous study in which neutrophils in aged mice were sequestered in the lung interstitium or

vasculature at 24 hours after burn injury [31]. Consistent with our previously published studies [31], lung neutrophil numbers did not differ between young sham and young burn mice 24 hours post injury (Table 1). These observations were confirmed by flow cytometry of whole lung cell suspensions. However, instead of using anti-MOMA-2 antibody for alveolar macrophages, anti-F4/80 antibody was used, since it detects circulating monocytes as well [33-35]. As shown in Table 1, while neutrophils (Gr-1^{hi} F4/80⁺ cells) in the lungs of young, burn-injured mice were similar to sham-injured animals, those from aged, burn-injured mice were 6 times greater than sham controls and young burn animals (p < 0.05).

We then sought to determine if neutrophils from either young or aged mice transmigrated and persisted in the alveolar space following burn trauma. Lungs were lavaged and BAL fluid was analyzed by flow cytometry (Table 1). As expected, BAL cells of both young and aged sham-injured mice were predominantly macrophages (F4/80⁺ Gr-1⁺ cells) and did not differ between treatment groups (data not shown). Neutrophils (Gr-1^{hi} F4/80⁺ cells) comprised approximately 4% in the young and 7% in the aged of cells recovered from BAL as determined by flow cytometry. Interestingly, the proportion of neutrophils in the BAL decreased in both young and aged mice after burn, although this was only statistically significant in the aged burn-injured mice compared to age-matched controls (p < 0.05). Together, these data indicate that the neutrophils are sequestered in the lung interstitial and/or vascular space of aged mice 24 hours after injury, but are absent in the alveolar space.

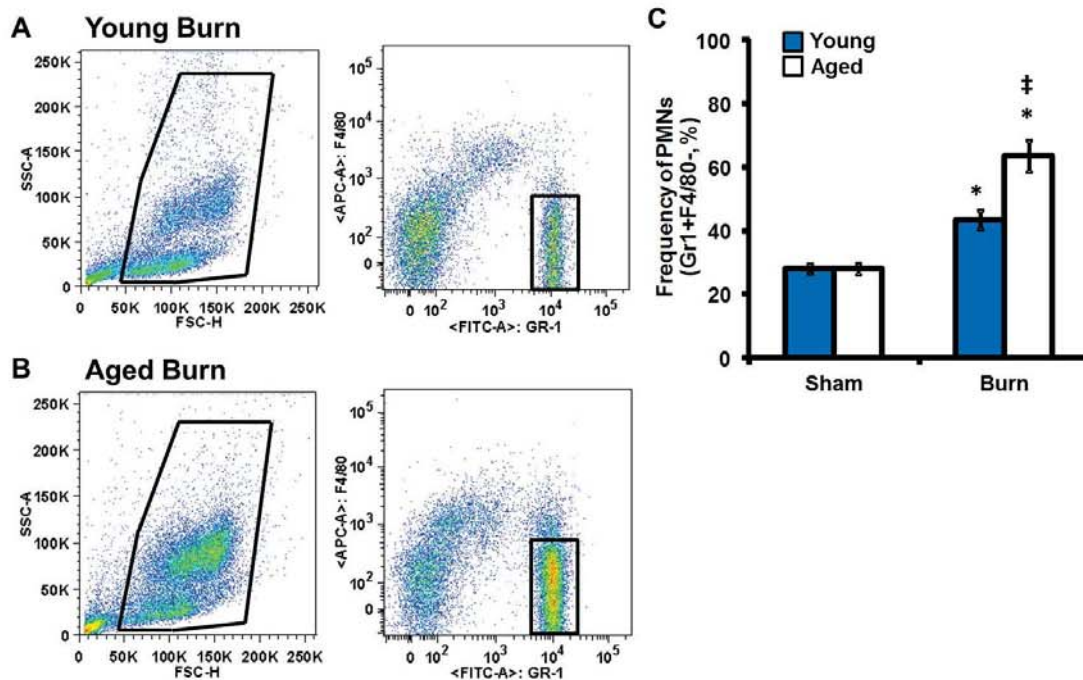


Figure 1. Peripheral blood neutrophil profile. Peripheral blood cells were stained with anti-Gr-1 and anti F4/80 antibodies and analyzed by flow cytometry. Representative live and neutrophil (Gr-1⁺F4/80⁺) for young (A) and aged (B) mice. (C) Total neutrophil frequency in whole blood. Data are shown as mean ± SEM. N = 8-15 mice per group; *p<0.05 compared to sham controls; ‡p<0.05 compared to young burn by one-way ANOVA.

Circulating neutrophil numbers and CXCR2 expression

Considering the heightened accumulation of neutrophils in the lungs of aged mice, we sought to examine the peripheral blood neutrophil population as circulating numbers of neutrophils or altered expression of the chemotactic receptor, CXCR2, on neutrophils may contribute to these observations (Figure 1). Following burn injury, both young and aged mice exhibit elevated percentages of circulating neutrophils in relation to sham controls (Figure 1A-C, p<0.05). However, aged mice demonstrate a ~20% increase in peripheral blood neutrophils as compared to young burn mice (Figure 1A-C, p<0.05).

As CXCR2 mediates the neutrophil chemotactic response to KC, previously shown to be significantly elevated in lungs of aged mice 24 hours post injury [31],

we sought to determine the frequency and mean fluorescent intensity (MFI) of CXCR2 expression in peripheral blood neutrophils (Figure 2). Interestingly, following burn injury there is a reduction in both the percentage of neutrophils expressing CXCR2 and the magnitude of CXCR2 expression in aged mice (Figure 2A-C, p<0.05). These data suggest that aging may alter the CXCR2 axis in the setting of trauma. Moreover, the prolonged elevation of KC in the lungs of aged mice at 24 hours post burn [31] may contribute to the observed downregulation of CXCR2 and alter neutrophil migration.

CXCR2-mediated neutrophil chemotaxis

Others have shown that pretreatment of neutrophils with various inflammatory stimuli can inhibit migration through activated endothelium *in vitro* as a result of

CXCR2 desensitization [6]. Coinciding with these studies, a basal pro-inflammatory state has been observed in the setting of advanced age and these mediators can remain elevated in aged mice in trauma models [14, 31, 36, 37]. Given the results of the previous study, we hypothesized that a migratory defect in neutrophils from aged mice may play a role in neutrophil accumulation in the lungs of aged mice after acute burn injury. Chemotaxis of isolated peripheral blood neutrophils from uninjured young and aged mice was observed in a transwell system in response to KC. This particular chemokine was chosen because our previous data indicated that there was an age-related increase between levels of KC lung homogenates and numbers of neutrophils in lung sections after burn injury [31][1]. Since pulmonary levels of MIP-2 in previous studies did not show age differences, this chemokine was not assessed. In the absence of any stimulant, migration

of neutrophils from aged mice was significantly higher compared to young mice (Figure 3, $p < 0.05$). In the presence of physiologic doses of KC, neutrophils from young mice had a robust response compared to unstimulated controls ($p < 0.05$). However, no chemotactic response was generated in neutrophils from aged mice in response to the same increasing doses of KC. Figure 3B shows the same data re-expressed as percent control to obviate the differences in trends between the two age groups. These data suggest that neutrophils from uninjured aged mice exhibit hyperchemokinesis, but impaired directional migration in response to a stimulus. The lack of stimulus-mediated motility may be due to defects in migratory mediators downstream of CXCR2 [17, 30, 38, 38].

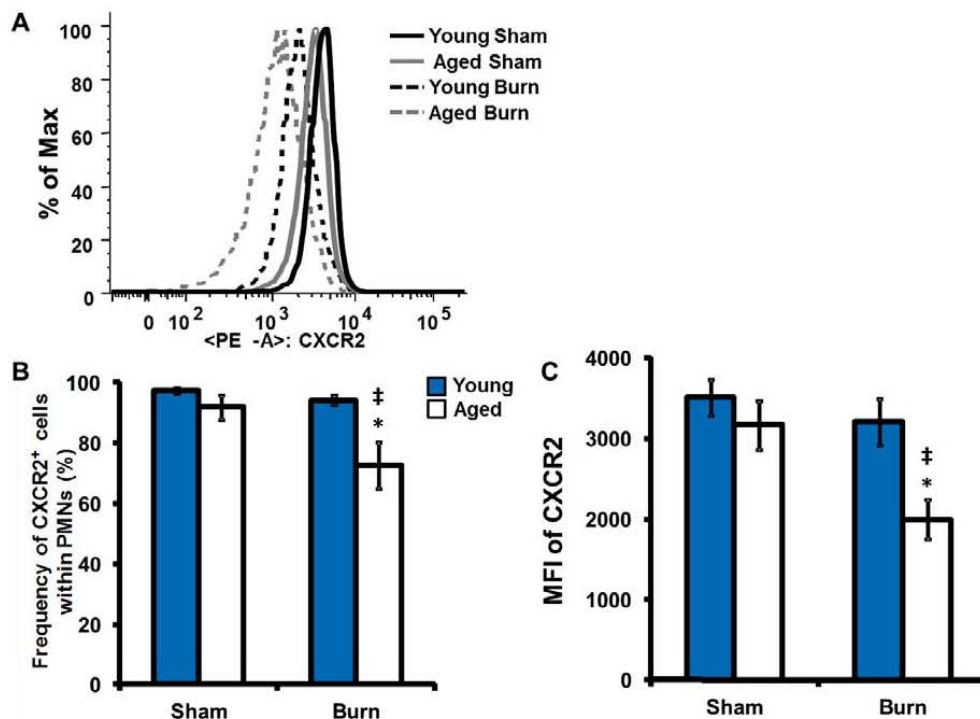


Figure 2. Peripheral blood neutrophil CXCR2 expression. Peripheral blood cells were stained with anti-Gr-1, anti F4/80 antibodies and anti-CXCR2 antibodies and analyzed by flow cytometry. (A) Representative histogram of CXCR2 neutrophil population with young sham- thick black, aged sham- thick gray, young burn- dotted black and aged burn- dotted gray. (B) Frequency of CXCR2⁺ cells within the neutrophil population; young- dark gray bars and aged- white bars. (C) Mean fluorescent intensity of CXCR2 within CXCR2⁺ neutrophils. Data are shown as mean \pm SEM. N = 8-15 mice per group; * $p < 0.05$ compared to sham controls; ‡ $p < 0.05$ compared to young burn by one-way ANOVA.

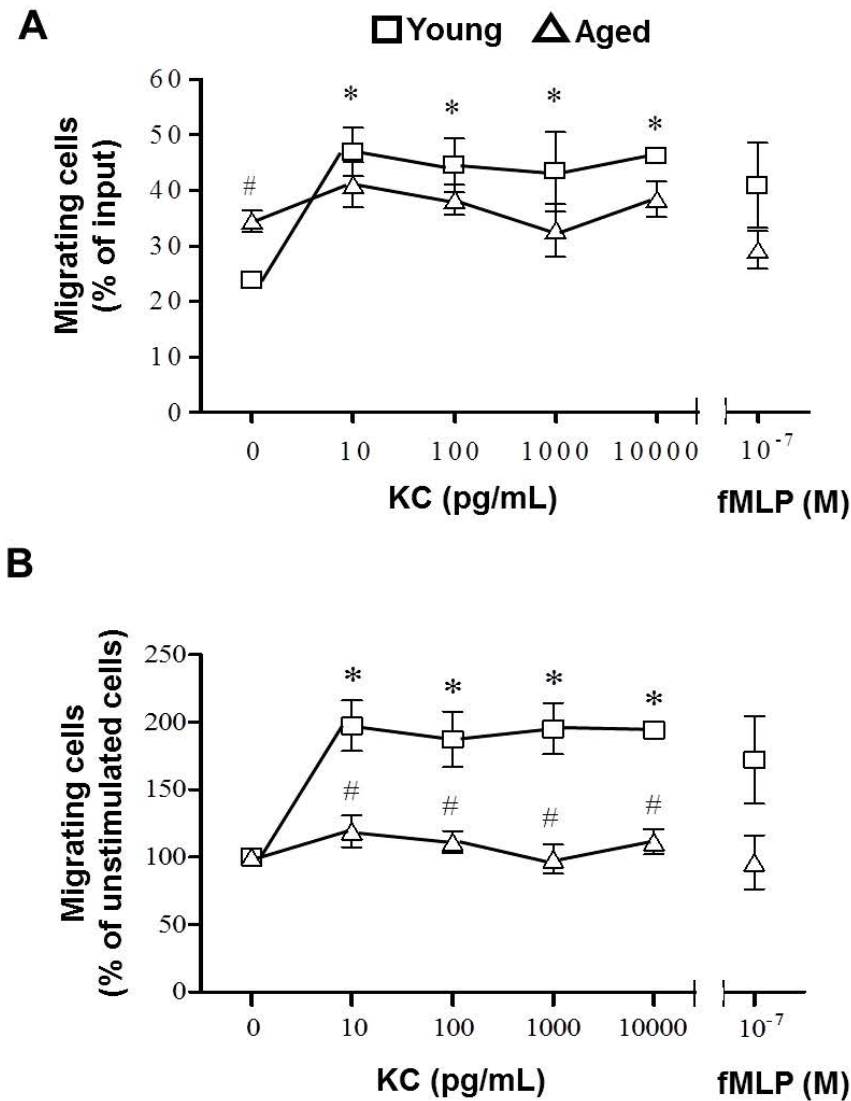


Figure 3. *In vitro* neutrophil chemotaxis assays. Peripheral blood neutrophils isolated from young (squares) and aged (triangles) mice were tagged with CellTracker Green and incubated with varying concentrations of KC in a transwell plate for 1 hour at 37°C. fMLP was used as a positive control. Migrating cells are expressed as (A) % of input (fluorescence of migrated cells/fluorescence of input \times 100) or (B) % of unstimulated cells (% input / % input of unstimulated cells \times 100). Data are represented as mean \pm SEM. N = 3-6 mice per group; * p <0.05 compared to young unstimulated control; # p <0.05 compared to young at the same chemokine dose by Student's t -test.

Table 2. Peripheral blood neutrophil adhesion molecule expression after burn ^a

	CD62L		CD11b	
	Sham	Burn	Sham	Burn
Young	702 ± 44	501 ± 12 *	3404 ± 232	3328 ± 206
Aged	670 ± 56	525 ± 13 *	3427 ± 259	3511 ± 398

^a CD62L and CD11b expression on peripheral blood neutrophils (F4/80⁺ Gr-1⁺ cells) from young and aged mice at 24 hours after sham or burn injury was determined by flow cytometry. Data are shown as mean fluorescent intensity ± SEM. N = 6-13 mice per group; *p<0.05 verse young sham by one-way ANOVA. Other data are not significant.

CD62L and CD11b expression on peripheral blood neutrophils

Activation of CXCR2 helps facilitate upregulation of selectins and integrins that mediate loose and firm endothelial adherence and transmigration. Elevation of neutrophil selectins or integrins may translate into increased neutrophil adherence to the vasculature, while the observed downregulation of CXCR2 and lack of neutrophil chemotaxis may prevent transmigration in response to apical KC levels. Thus, we sought to examine neutrophil levels of CD62L and CD11b expression by flow cytometry (Table II). Despite the heightened pulmonary neutrophil congestion and impaired directional motility observed in aged mice, no difference in the frequency of CD62L and CD11b positive neutrophils was seen (data not shown). While the MFI of CD62L was reduced in both young and aged mice subjected to burn injury compared to young sham, no significant difference was noted in CD62L and CD11b expression in aged, burn-injured mice as compared young, burn-injured mice. These data suggest that alterations CD62L and CD11b expression does not contribute to the exacerbated pulmonary inflammatory response or impaired chemotaxis found with advanced age.

Pulmonary vascular ICAM-1 expression in response to burn injury

While no differences in CD62L and CD11b were detected, others have shown that endothelial ICAM-1 is important in neutrophil recruitment to the lungs after injury [12, 39-42]. To further investigate this in our model, lung sections were stained for ICAM-1, expressed by both pulmonary endothelial and epithelial cells, and PECAM-1, a constitutively expressed endothelial marker [43]. Colocalization of ICAM-1 and

PECAM-1 was considered to be endothelial ICAM-1 expression (Figure 4A). ICAM-1 expression on the pulmonary vasculature in aged, burn-injured mice was increased greater than 3-fold compared to sham controls (p<0.05), while no differences were detected in the lungs of young, burn injured mice (Figure 4B). This suggests that pulmonary vasculature in aged mice may more readily bind activated neutrophils that are passing through the lung, subsequently leading to their retention.

DISCUSSION

In summary, the data presented in this study show for the first time that neutrophils are retained in the lung vasculature and/or interstitial space of aged mice after acute burn injury and do not transmigrate into the alveolar space. This process is related, in part, to an altered pulmonary endothelial adhesion molecule profile and decreased capacity for neutrophil chemotaxis in aged mice compared to young mice at 24 hours after burn. Similar features have been shown in young animals within a few hours of injury; however, this response is transient and return to sham levels by 24 hours [42, 44, 45]. The present findings are compounded by the elevated numbers of neutrophils in the peripheral blood, which may promote enhanced congestion in the absence of adequate clearance in aged mice. The difference in pulmonary endothelial adhesion, as well as neutrophil number and function, in aged mice may contribute to the delayed resolution of the pulmonary inflammatory response as seen in our previous study [31] and might contribute to worse outcomes for this group. Taken together, these observations are important in understanding the pathogenesis of burn injury in aged individuals and suggest that prolonged inflammation may be responsible for the various complications seen in this population.

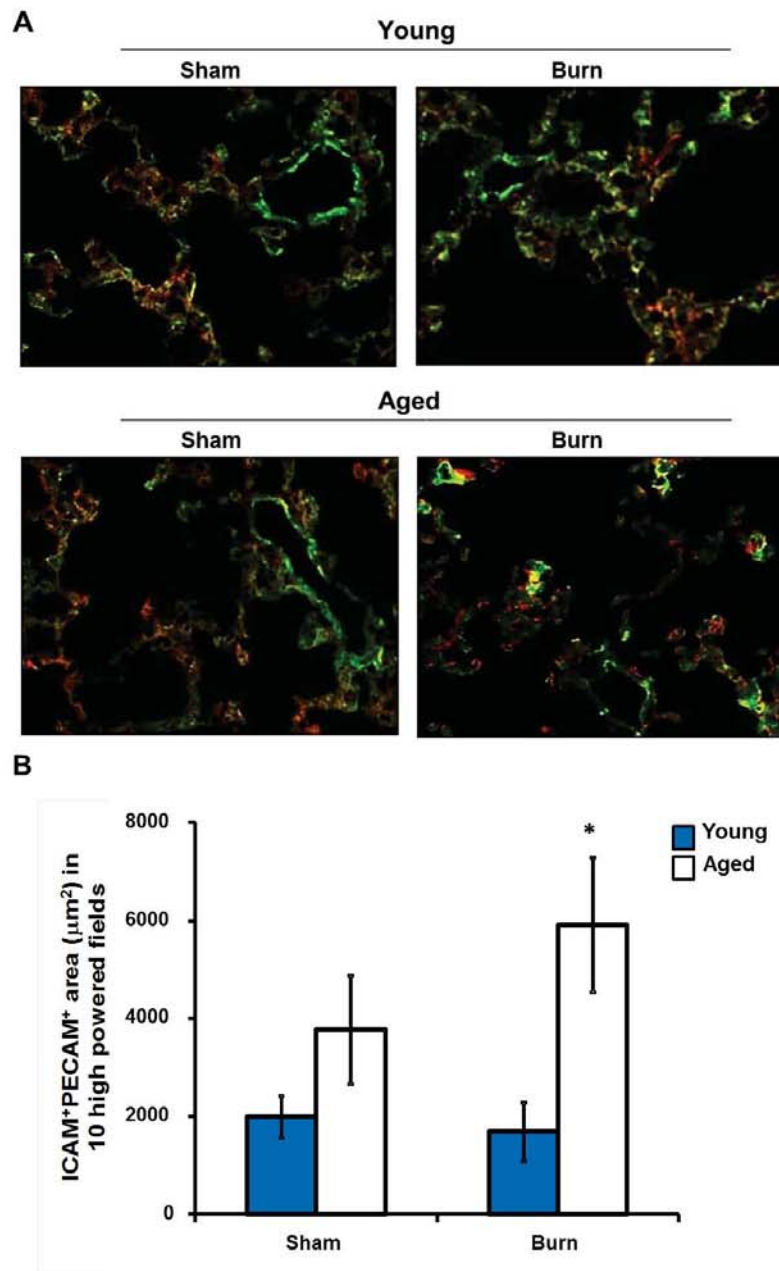


Figure 4. Pulmonary endothelial ICAM-1 expression after burn. Lung sections immunostained with anti-ICAM-1 (red) and anti-PECAM-1 (green) antibodies. (A) Representative images are shown from young and aged mice at 24 hours after sham or burn injury at 200x magnification. (B) Total ICAM-1⁺PECAM-1⁺ area (μm^2) in ten high powered fields (400x). Data are shown as mean \pm SEM. N=8-13 mice per group; * $p < 0.05$ compared to young burn by one-way ANOVA.

A prominent theory known as “inflamm-aging” suggests age-associated problems are related to elevations in circulating pro-inflammatory mediators in the absence of clinically detectable disease [46-48]. These findings have been corroborated by others in BAL from aged humans, in which interleukin (IL)-1 β and IL-6 were elevated in the absence of injury or disease [49-51]. Previously, we have established that relative to young, traumatic injury in aged mice exacerbates this basal pro-inflammatory state at early time points [14, 31, 36]. Of note, a study by Luu et al. demonstrated that preincubation of neutrophils with various chemokines inhibits transmigration through an activated endothelial monolayer *in vitro* [21][6], suggesting that a persistent pro-inflammatory stimulus can lead to neutrophil dysregulation. Moreover, these neutrophils were able to undergo firm adhesion, but were not able to transmigrate across the endothelial layer. Interestingly, this finding was related to desensitization of the neutrophil chemotactic receptor CXCR2. Currently, our study found that following burn trauma, neutrophils from aged mice exhibit decreased expression of CXCR2. These findings recapitulate observations from human studies in which CXCR2 was downregulated in trauma patients [52, 53]. This reduction may be due to the elevated levels of circulating cytokines and chemokines [31], either through receptor desensitization or receptor ligation and uptake [38, 54, 55]. Regardless, once these neutrophils reach the pulmonary circulation, diminished CXCR2 activity may impair their ability to respond to apical chemokines to mediate transmigration through the endothelium [21, 29].

One interesting finding from this study is that peripheral blood neutrophils from aged mice have an increased ability for random migration, or chemokinesis, compared to those from young mice, as shown by others [48, 56]. Our results indicate that neutrophils from aged mice have a heightened basal level of random migration; however, their ability to respond directionally to a specific stimulus, such as KC, is impaired. While there is some contention in the literature, this idea of heightened basal neutrophil activation in aged mice parallels observations in human studies [57-59]. Advanced age has been associated with increased intracellular Ca²⁺ and G-protein coupled receptor kinase activation [58, 60, 61], both of which modulate neutrophil chemotactic pathways [38, 62-64][7-9]. Similar to the current study, others have shown that neutrophils from aged mice are incapable of generating a peak response once they are exposed to a secondary stimulus, such as direct KC stimulation, live bacteria, or burn injury [58, 61]. Further studies characterizing the direct role of CXCR2 in our model will potentially elucidate this mechanism.

In addition to the chemotactic role of CXCR2, reports indicate that the ability of neutrophils to signal through CXCR2 is important for adherence to and migration through endothelial layers [19, 21, 65, 66]. Activation of CXCR2 leads to upregulation and clustering of selectins, like CD62L, and integrins such as Mac-1 (CD11b/CD18), lymphocyte-function associated antigen-1 (LFA-1: CD11a/CD18) and very late antigen-4 (VLA-4: CD49a/CD29) [24-26]. Specifically, signaling via CXCR2 and other chemokine pathways induce a conformational change in CD11b, allowing it to form a stronger interaction with ICAM-1 on endothelium and undergo adhesion [42, 66, 67]. Due to the differences in CXCR2 expression, we hypothesized that age may be associated with differences in adhesion molecules CD62L and CD11b. However, levels of CD62L and CD11b were found to be comparable between young and aged mice, pre- and post-burn. Additional studies are required to examine the role of CD11b clustering in this model, as well as other neutrophil integrins such as CD11a/CD18 and CD49a/CD29, to determine whether aging alters neutrophil adhesion molecules that promote firm adhesion to the pulmonary endothelium.

Another component critical to neutrophil adhesion and subsequent transmigration is the expression of various adhesion molecules by the pulmonary vasculature. Here, we demonstrate that aged mice have increased expression of pulmonary endothelial ICAM-1 and this correlates with elevated neutrophil numbers within lung tissue. Previous studies in combined trauma models have shown that loss of ICAM-1 decreases neutrophil recruitment into the lung [68], suggesting that targeting ICAM-1 may ameliorate the neutrophil congestion seen in our study. Moreover, elevated levels of pro-inflammatory mediators can activate endothelial cells, resulting in upregulation of ICAM-1 and other adhesion molecules and a pro-adherent endothelium [17, 69, 70]. We have previously demonstrated that following burn, aged animals have elevated circulating levels of pro-inflammatory cytokines and chemokines which likely promotes upregulation of ICAM-1. Moreover, human studies have revealed the cross-linking of ICAM-1 stimulates production of IL-8, a human neutrophil chemokine [71]. This upregulation in IL-8 may be to further enhance neutrophil chemoattraction, but may also aid in the neutrophil trans migratory response to apical chemokines. Additional investigation into vascular adhesion molecule-1 (VCAM-1) signaling, as well as other adhesion molecules such as VCAM-1, will further elucidate the impact of age and trauma on lung endothelium.

Taken together, our data suggest that a combined defect in CXCR2 signaling and elevated ICAM-1 promotes neutrophil congestion in the lungs of aged

mice following burn trauma. The elevated ICAM-1 may immobilize these neutrophils within the vasculature, but their inability to adequately respond to local chemokines may prevent their eventual diapedesis and clearance. We propose that the inflammatory environment of the aged animal primes neutrophils and renders them incapable of responding appropriately to an inflammatory insult, such as burn injury. Moreover, the exacerbated pro-inflammatory environment in aged mice following burn trauma may promote an adherent phenotype within the pulmonary vasculature, further compounding the observed neutrophil defects. Understanding the mechanism involved in this process is important for identifying potential targets of therapy with the ultimate goal of improving outcomes for aged patients sustaining a burn or other traumatic injury.

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